

# The Quarterly Journal of Microscopical Science

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*Joint Editors*

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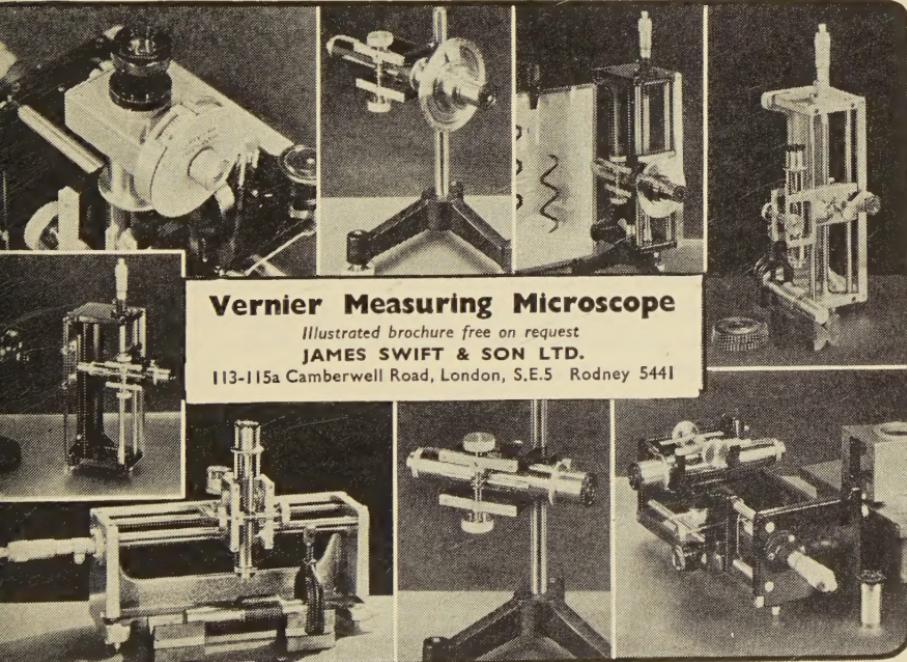
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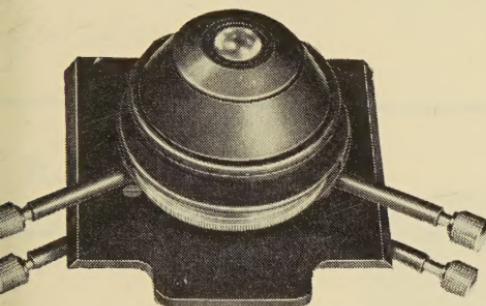
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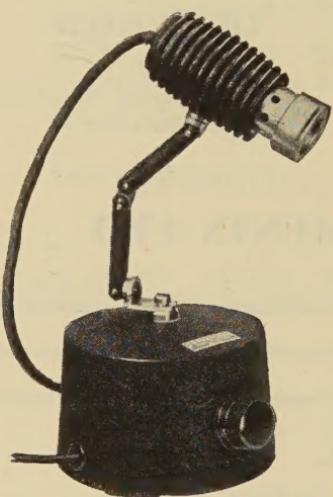
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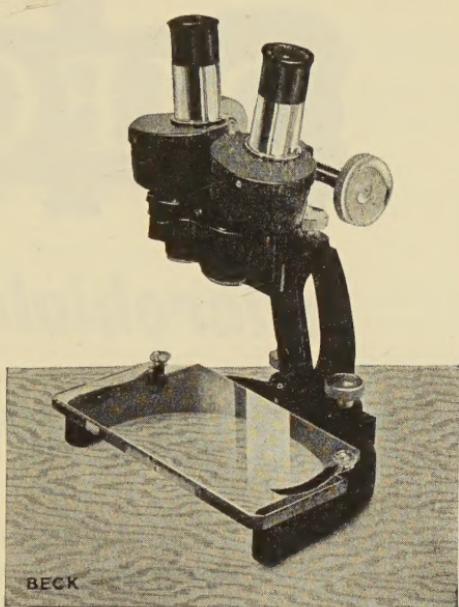
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## The Masked Lipids of Nuclei

By J. CHAYEN, P. B. GAHAN, and L. F. LA COUR

*From the Imperial Cancer Research Fund, Department of Pathology, the Royal College of Surgeons, Lincoln's Inn Fields, London, W.C. 2; and the John Innes Horticultural Institution, Bayfordbury, Hertford, Herts.)*

With one plate (fig. 3)

### SUMMARY

The histochemistry of masked lipids of chromosomes has been investigated with the aid of paper chromatographic procedures. At least three tightly bound associations of phospholipid with protein have been identified in calf thymus nucleohistone. Hence it seems likely that the 'spurious' reactions of nuclei and of nucleohistone, especially after extraction with lipid solvents, is a true indication of the increased availability of these closely linked phospholipids. Moreover, not more than about 10% of the material extracted by hot pyridine from calf thymus was fatty, the rest being water-soluble. After treatment of plant cells with hot trichloroacetic acid had produced increased staining with methods for demonstrating lipids, fatty matter could be extracted from the tissues. This demonstrates that the cells which gave the 'spurious' reaction did contain lipid. Hence it seems probable that extraction with solvents does not remove all lipids but may make those that remain more available for staining, so giving rise to what has been considered to be a spurious reaction.

The nature of the binding of lipid to protein and the relevance of such complexes to histochemistry and the composition of nuclei are discussed.

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### INTRODUCTION

FOR many years histologists have considered that some lipids may occur in such a form that they cannot be demonstrated until some pathological condition or special histological treatment reveals them. In order to explain

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this visualization of lipids it is necessary to assume either that the lipid is present in a form that is available for staining but is so finely dispersed that it cannot be seen, or that it is bound to other material in such a way that colouring agents for lipids cannot dissolve in it. Berg (1951), in a careful study of masked lipids of many types of tissues, tended to equate 'masked lipid' with that fatty matter which is present in very fine dispersion. During the fatty degeneration of the cells these finely divided particles were believed to coalesce to form resolvable droplets. Berg therefore considered that because of its fluorescence, 3:4-benzpyrene could be used to demonstrate the presence of this type of masked lipid and his results with this fluorochrome are impressive. In the opinion of the present authors, however, such fatty matter should be designated 'finely dispersed lipid' since it has no 'mask' or masking agent associated with it.

The possibility that lipids might be unavailable to stain because of the presence of a true mask of protein, or other substance, has been suggested tentatively by Grundland and Bulliard (1938) (in Lovern, 1957). The purpose of the present investigation was to test the validity of the concept of 'masked lipids' in histochemistry, particularly of phospholipids.

Phospholipids may be identified by Baker's acid-haematein and pyridine extraction tests. Baker (1946, 1947), after a detailed and careful study, concluded that phospholipid was present in tissues if the acid-haematein test was positive under normal conditions, but negative after prior extraction with pyridine. Although such results were obtained in some parts of cells, others, notably the nuclei, stained more intensely after treatment with pyridine. Baker, although aware of the possibility that this might be due to the presence of masked lipids, was forced on reasonable grounds to conclude that, until the occurrence of such lipids was proved, it was not possible to deduce their presence from these data (also see Cain, 1950). Hence the only permissible conclusion that could be reached was that, where there was an increase in staining after the pyridine extraction test, nothing could be said about the presence or absence of phospholipids. Thus it was impossible to decide if these substances occurred on interphase or mitotic chromosomes. Moreover since Baker (1946) had shown that nucleohistone from calf thymus yielded 'spurious' reactions for phospholipid with his tests, it seemed likely that this material was the 'interfering substance' present on the chromosomes.

Thus it was considered advisable to reinvestigate the phenomenon of masked lipids in histochemistry by means of the new methods which are now available (e.g. see Lovern, Olley, Hartree, and Mann, 1957) for the identification of very small quantities of phospholipids. Three questions had to be considered: First, is nucleohistone an 'interfering substance', or does it contain closely bound (masked) lipids? If it were the former, then it would be impossible to show the presence of phospholipids in nuclei by histochemical means. If it contained bound lipids, however, these would be expected to occur also in intact nuclei. Secondly, does pyridine extract only fatty material or might it also remove a substance which could mask strongly bound lipid?

Thirdly, in a tissue in which lipid has been apparently unmasked, is there any evidence that fatty substances are present?

## MATERIALS

Nucleohistone was prepared from calf thymus in two different ways. In the first (Mirsky and Pollister, 1947), the thymus was homogenized in a 1 M solution of sodium chloride in an M.S.E. homogenizer, filtered, and the dissolved deoxyribonucleohistone precipitated out of solution by diluting the salt concentration to about 0.14 M. It was redissolved in the stronger saline and then reprecipitated by dilution. The second method, that of Doty and Zubay (1956), is a modification of the procedure of Shooter and others (1954) for preparing nucleohistone of very high molecular weight. For this, 200 g of frozen calf thymus were placed in a Kenmix '55' blender, after removal of most of the connective tissue. It was just covered with a saline-versene solution (0.075 M NaCl + 0.024 M Na versenate at pH 8.0) containing 2 ml lauryl alcohol (octan-2-ol) and homogenized for 3 min until an even homogenate was obtained. The volume was made up to 1 litre with the saline-versene solution and homogenized for a further 10 min. The solution was filtered through gauze which had been washed with saline-versene, and spun in 250 ml buckets in an International Refrigerated Centrifuge at 2,800 r.p.m. for 20 min. At the end of the spinning the supernatant fluid was discarded. The sediment was resuspended in 500 ml of saline-versene and 1 ml of octan-2-ol, and spun for 10 min at 2,800 r.p.m. The supernatant was discarded and the sediment resuspended in 500 ml of saline-versene solution and 1 ml octan-2-ol. This was spun at 3,000 r.p.m. for 10 min. This last process was repeated three times more. The final precipitate was believed to be pure nucleohistone (58.68 g wet weight).

## METHODS

### *Separation of water-soluble materials that may be mixed with lipids.*

The method of Folch and others (1951b) was followed. The crude matter was dissolved in a mixture of one volume of methanol to two or three of chloroform and this solution was pipetted, drop by drop, into a column of water in a 2-litre measuring cylinder. The drops fell into a small dish which acted as a false bottom to the cylinder. The liquid was allowed to stand, under the water, at least overnight in a cold room. The methanol passed rapidly into the water, both from the individual drops and from the accumulated fluid, causing severe currents by which water-soluble material escaped into the water. The residual solution in chloroform was then removed, three volumes of methanol were added, and the process was repeated with a fresh column of water. The matter which remained in the chloroform, plus any present at the chloroform-water boundary, was regarded as lipid or as substances probably combined with lipids. The efficiency of this procedure in removing ultra-

violet-absorbing substances which were dissolved in methanol-chloroform can be seen in fig. 2.

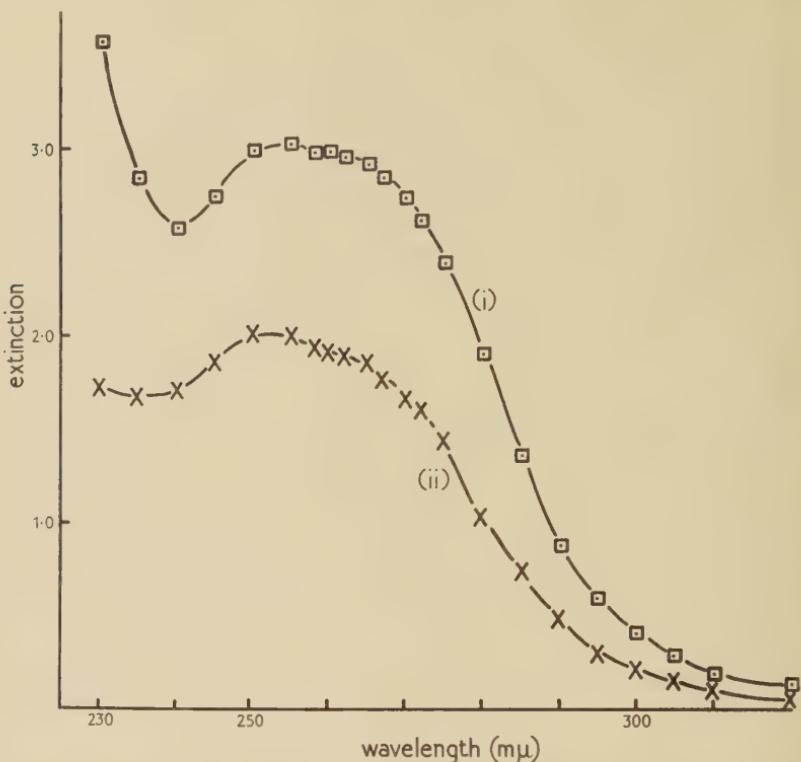


FIG. 1. Curve (i), the absorption curve of the formaldehyde-calcium after calf thymus had been fixed in it. A nucleotide-like peak at about  $260\text{ m}\mu$ , but including a protein-like shoulder at  $270\text{ m}\mu$  is noticeable. The fixative was diluted 10 times. Curve (ii), the ultraviolet absorption of the formal-calcium after the lipid-protein complex had been removed by shaking the used fixative with chloroform. Diluted 10 times. Curves (i) and (ii) are qualitatively similar; the differences in height are due to some material being precipitated out by the chloroform.

**Hydrolysis procedures.** Lecithins and cephalins, in the free state, can be hydrolysed by boiling for 2 h in a 0.5 N solution of potassium hydroxide in 96% ethanol under a reflux condenser or saponification column (see Lovren 1957). The unsaponifiable matter is removed from the cooled hydrolysate by shaking with ether. The fatty acids are not removed because they are present as the water-soluble potassium soaps; they are freed from the soaps, therefore, by acidifying to about pH 5.5 and are then removed by shaking again with ether. The remaining aqueous hydrolysate contains such components as choline, glycerophosphate, serine, and ethanolamine. During such hydrolysis there is some conversion of  $\alpha$ -glycerophosphate to the  $\beta$ -form (Dawson, 1957).

**Chromatographic methods.** The water-soluble components of the hydrolysate were concentrated and an 'Aglag' micrometer syringe was used to place measured volumes of it on to a line on a sheet of Whatman No. 1 filter paper.

known amounts of the pure substance were placed between these 'spots' of the hydrolysate. It was sometimes preferable to use filter paper that had been washed with hydrochloric acid (Hanes and Isherwood, 1949), as this gave

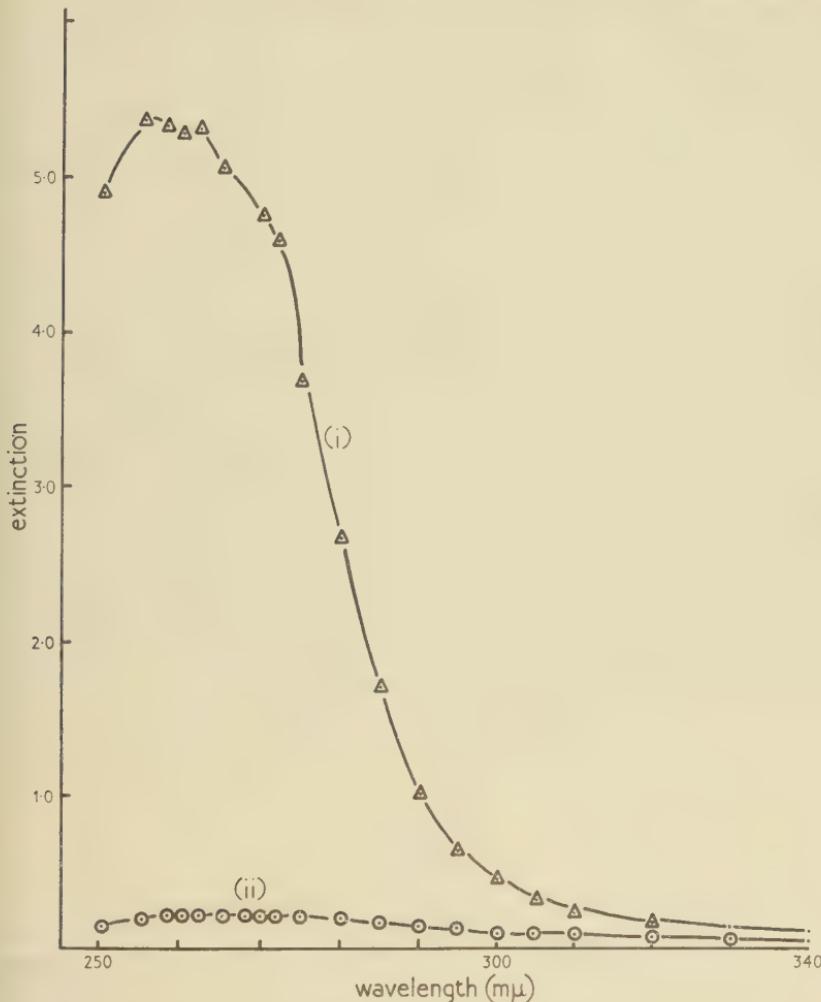


Fig. 2. Curve (i), the absorption of that part of the precipitate which was soluble in methanol-chloroform. The presence of substances absorbing maximally in the region of 260 and 275 m $\mu$  is noteworthy. Curve (ii), the absorption, in methanol-chloroform, of the lipoidal matter which remained after the procedure of Folch for the removal of water-soluble compounds. These two curves show the efficiency of this procedure in purifying lipoidal substances.

aner separations. Ascending chromatograms were then run, the solvent being 80% *n*-propanol.

When dry each chromatogram was studied to visualize a particular component of phospholipids. Choline was demonstrated by the method of Levene and Chargaff (1951), in which the substance is treated so as to produce an soluble phosphomolybdate. The free phosphomolybdate is washed out

of the paper and the insoluble material is reduced to yield an intense blue dye. The evidence that a particular coloration on the chromatogram represents choline is (a) that it produces an insoluble phosphomolybdate, and (b) that it has the same  $R_F$  value as the control spot for choline (see fig. 3). Glycerophosphate was visualized by the method of Burrows and others (1952), which demonstrates the presence of phosphoric acid. Again, the proof that the material which contains phosphate is  $\alpha$ - or  $\beta$ -glycerophosphate is obtained by comparing its  $R_F$  value with that of the control, run simultaneously on the same piece of paper. Serine and ethanolamine were identified by spraying with 0.2% ninhydrin in absolute *n*-butanol and heating for 10 min at about 110° C. This reaction is given by any free amino group. Inositol was sought by the method of Trevelyan and others (1950).

When quantitative estimates were required, three control spots containing different amounts of the known substance, and between these two different concentrations of the hydrolysate, were run. The areas of the developed spots were measured by planimetry. There is frequently a logarithmic relationship between the area of the spot and the amount of a particular material present (see Levene and Chargaff, 1951; also Fisher and others, 1948), so that the amounts of a substance present in the known volumes of hydrolysate were estimated from a graph of this relationship obtained from the controls.

In the present study sphingosine has not been estimated. The evidence concerning its occurrence, probably as sphingomyelin firmly bound to protein in the nucleohistone used in the present investigation, has been presented by Chayen and Gahan (1958).

*Histochemical and staining methods.* Lipids were demonstrated by the use of an alcoholic solution of Sudan black B, by Baker's acid haematein test, and by the orange G aniline blue method described by La Cour and Chayen (1958).

## RESULTS

### *The lipids of nucleohistone*

*Lipids in unprecipitated nucleohistone.* The nucleohistone was prepared by Doty and Zubay's method (1956). It was boiled, under a reflex condenser, for 2 h in ethanolic potash. Most of the material dissolved and the small sediment which remained was removed (*C*, see below). The hydrolysate was shaken with ether to remove the unsaponifiable matter and then acid was added to adjust the pH so as to free the fatty acids. As the pH approached pH 6, a pale yellow precipitate consisting of short adhesive threads began to form. The pH was brought to pH 5–6 and the precipitate was removed (*B*). The filtrate was shaken with ether to remove the fatty acids, the aqueous phase (material *A*) was concentrated and was examined by paper chromatography. This demonstrated the presence of a phosphate- and a choline-like moiety, both apparent

FIG. 3 (plate). A chromatogram demonstrating choline in sample *C*. The acid hydrolysate of *C* is in the second and fourth columns, measured from the left, and known amounts of choline are in the first, third, and fifth columns.

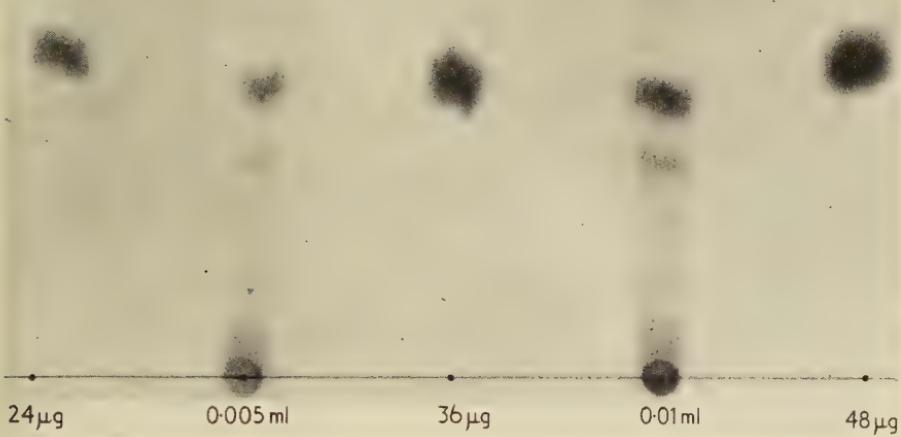


FIG. 3

J. CHAYEN, P. B. GAHAN, and L. F. LA COUR



ound to other substances since they did not run freely from the starting line. A spot which seemed to correspond to free  $\beta$ -glycerophosphate was found; no reducing sugars were observed.

The material (*B*), which had been dissolved by the ethanolic potash but which precipitated at between pH 5 and 6, was hydrolysed by boiling for 3 h under a reflux condenser, with 6 N hydrochloric acid. After shaking with ether to remove fatty acids, the aqueous phase was evaporated to dryness and the residue was dissolved in water for chromatography. One chromatogram was sprayed with ninhydrin and showed many unidentified spots.  $\beta$ -Glycerophosphate, some bound phosphate, and free choline were also demonstrated; no reducing sugars were detected.

The sediment (*C*) which remained after the original hydrolysis in ethanolic potash was boiled in 6 N aqueous hydrochloric acid for about 2 h and this hydrolysate, treated in the same way as was that of *B*, was studied by chromatography. Choline and glycerophosphate, both bound and free-running with the correct  $R_F$  value, were found. There was much ninhydrin-staining matter, with strongly positive regions or spots which had  $R_F$  values similar to those of serine and ethanolamine. No reducing sugars were detected.

TABLE I

Material	Hydrolysis treatment	Amount of material found in the hydrolysate	
		†glycero phosphate	†choline
nucleohistone' insoluble material, <i>C</i>	ethanolic potash	(mg.) +	(mg.) 37.1
	6 N hydrochloric acid for 2 h	327	24.4
precipitate, <i>B</i> , from the hydrolysis of 'nucleo- histone'	6 N hydrochloric acid for 3 h	26	61.2
<i>Total amounts</i>		353	122.7

† Only spots having the correct  $R_F$  value have been estimated.

Substance *C* resembles a lecithin in containing glycerophosphate and choline, but the former is present in excess of what would be found in a normal phosphatidyl choline. It is possible that some of the glycerophosphate was combined with serine or ethanolamine in a cephalin-like arrangement. The astonishing fact, however, is that if cephalins and lecithins were present in this fraction, they were not even dissolved by boiling alcoholic potash, which dissolves and degrades normal glycerophosphatides to their component moieties. Substance *B*, on the other hand, dissolved in the ethanolic potash but was precipitated at a more acid pH. The results of its hydrolysis by acid suggest that it too contained either a lecithin with an excess of glycerophosphate or a mixture of lecithins and cephalins. The fact that they were not degraded by the alkaline hydrolysis and were precipitated on acidification,

suggests that they may occur as a lipid-protein complex. The results obtained with ninhydrin support the view that protein may be present in this substance.

It would seem, therefore, that this nucleohistone contained four lipid-like components: those of fractions *B* and *C*; the matter in fraction *A* which yielded the free glycerophosphate after alkaline hydrolysis only; and the bound lipid-like matter of fraction *A*. It is not known whether the free glycerophosphate of *A* was derived from the lipid of the heterochromatic regions of the nuclei (Chayen and others, 1959), or from the bound matter. It is likely that the lipids of *B* and *C* were bound tightly to some other substance, probably protein, in such a way that they were protected from solution and from hydrolysis unless the protein itself were hydrolysed. This is of concern because of its significance in histochemistry, where a binding which is so resistant would be expected to protect the lipid from extraction in simple solvents.

It would seem desirable to make some estimate of the proportion of lipid-like matter in this nucleohistone. No precise value can be obtained from the present data since only the hydrolysis products of the lipids were determined and fatty acids have not been estimated. This is immaterial for our investigation, the purpose of which is not to identify the lipids exactly but rather to see whether lipid components occur in sufficient quantity to affect histochemical reactions.

The data in table 1 can be evaluated in at least two ways.

(i) According to Chayen and Gahan (1958), sphingomyelin could account for some 6% of this nucleohistone. Thus if their assumptions are correct 168 mg of the present sample of nucleohistone (dry weight 2.81 g) could be sphingomyelin. If it is assumed that the fatty acid associated with this material is lignoceric acid (Lovern, 1957) the molecular weight of the sphingomyelin would be 844 and hence it follows that 168 mg would liberate about 24 mg of choline. The total amount of choline recovered was 122.7 mg (table 1), so that some 98 mg remained to be accounted for.

If this residual choline was present in a phosphatidyl choline which contained  $C_{22}$  acids as both fatty acid moieties, this choline plus about 173 mg of the glycerophosphate would have been derived from about 0.85 g of this lecithin. There is still an excess of 180 mg of glycerophosphate. If it is assumed that this is present only as glycerophosphate (perhaps bound to protein), the total lipid content of this nucleohistone would be 0.85 g of phosphatidyl choline, 0.17 g of sphingomyelin, and 0.18 g of glycerophosphate, totalling 1.2 g out of the original 2.81 g of 'nucleohistone'. Thus lipid-like materials would comprise some 46% of the 'nucleohistone'.

(ii) This figure must be considered the upper limit and seems absurdly high. A lower limit can be calculated by assuming that sphingomyelin (168 mg) is present and that no fatty acids occur. The rest of the choline (98 mg) might then be combined with 173 mg of the glycerophosphate to yield 271 mg of glycercylphosphoryl choline (see Dawson, 1957); since this compound is water-soluble it would have to be linked to some other moiety, such as fatty acids or proteins. Similarly, the residual glycerophosphate (180 mg) must be

sesed as glycerophosphate, which may be bound to protein. Hence the total amount of lipid-like material is 619 mg, which constitutes about 28% of the 'nucleohistone'. This seems to be a more reasonable figure since no estimates were made of the fatty acid content. Although a lipid, biochemically, defined as possessing fatty acids, the glycerophosphate (or the sphingosine) esterites may be taken as the histochemical markers for phospholipids.

#### *Lipids in reprecipitated nucleohistone*

In a somewhat preliminary study this nucleohistone was boiled in ethanolic potash for 2 h under a reflux condenser. Subsequently any unsaponifiable matter and fatty acids were removed and the hydrolysate was concentrated to a small volume for chromatography. Some glycerophosphate was found, possibly corresponding to the phospholipid of heterochromatic regions (Chayen and others, 1959). No free choline was found but much choline-like material, yielding insoluble deep blue salts with phosphomolybdic acid, was observed. Most of the choline-like matter had not moved from the starting line and correspondingly, phosphate-containing material occurred at the same points on the chromatogram. It seems likely, therefore, that even the reprecipitated nucleohistone contained the phospholipid of the heterochromatic regions plus other, alkali-stable lipids.

Hence it may be concluded that when nucleohistone stains positively for phospholipid, its reaction is probably a true one. Moreover, although it is possible that the pyridine extraction test may remove the surface phospholipid from the heterochromatic regions of nuclei, it may also unmask the tightly bound phospholipids (see below) which will then be free to give true positive reactions with the acid haematein test.

#### *The effect of extraction with pyridine*

When plant roots were fixed in formaldehyde-calcium and then hardened either in dichromate or in Lewitsky's (1931) fluid and embedded and sectioned, they stained very weakly with methods for phospholipids (see La Cour and others, 1958), but if after fixation they were treated with pyridine at 60° C for 24 h, all parts of the cells, but particularly the nuclei, stained vividly. A similar great increase in staining was obtained with calf thymus which had been treated with hot pyridine. If the pyridine removed only lipoids, the increased staining was most probably due to a spurious reaction of the treated tissue, but if it extracted other material it was possible that this was the agent which masked a very stable lipid.

Pieces of calf thymus were placed in pyridine at 60° C for 24 h, after which the pyridine was decanted, centrifuged to remove cell debris, and evaporated to dryness. The dried residue was weighed, shaken with methanol-chloroform, and left at 60° C for 2 h in a corked container to dissolve lipid-like substances. The solution was centrifuged to remove insoluble material and then subjected to the Folch procedure and left under water at about 4° C for 3 h. The soluble matter did not seem to be lipoidal and did not dissolve in hot

ethanol-ether. The material which remained soluble in the chloroform after the Folch procedure, plus insoluble matter floating on its surface, were considered as fatty substances and were collected together, dried, and weighed. They corresponded to only about 8% of the total residue obtained from the pyridine (see table 2).

It is very likely that, under the conditions of this test, the extraction of lipids by pyridine was incomplete. However, the experiments do answer the question posed, namely does pyridine extract only fatty material or might it also remove a substance which could be masking strongly bound lipid?

TABLE 2

<i>Wet weight of thymus</i>	<i>Dry weight of material extracted by pyridine</i>	<i>Weight of extracted matter that was insol. in meth-chl.</i>	<i>Weight of 'lipid' in chloroform after the Folch procedure</i>	<i>Weight of water-sol. material after the Folch procedure</i>	<i>Percentage 'lipid' of extracted matter</i>
g	g	g	g	g	(%)
7.1543	0.113	0.0558	0.0099	0.0473	8.76
10.1988	0.1535	0.1188	0.0070	0.0277	4.56
11.5063*	0.1507	0.1201	0.0105	0.0201	6.97
7.7872*	0.1406	0.0996	0.0190	0.0220	13.52

\* The material extracted in pyridine was not heated with methanol-chloroform in these experiments.

### *The demonstration of lipid in tissues which showed the 'spurious' reaction*

'Spurious' reactions for phospholipids were obtained in plant root cells after fixation in formaldehyde-calcium and treatment with hot pyridine, with 5% trichloroacetic acid at 90° C. for 15 min, or even with hot ethanol-ether. Each of these procedures greatly increased the staining for lipids throughout the cells but particularly in the nuclei. Since the action of trichloroacetic acid is fairly well known, it was thought advisable to examine the tissues after treatment with this liquid to see if the staining reaction was indeed spurious and whether lipid-like matter was present which might account for the reaction.

Roots of *Trillium grandiflorum* and *Vicia faba* were fixed in formaldehyde-calcium, and those of *Scilla campanulata* in 45% acetic acid for 5 h. A few roots from each batch were removed for cytological examination to ensure that only the nucleoli stained appreciably for lipids. The rest of the roots were treated with 5% trichloroacetic acid at about 90° C for 15 min. Some were taken for cytological inspection to confirm that this procedure had induced strong lipid reactions in the nuclei and in the cytoplasm; these reactions were least marked in *Vicia* roots. The remainder were put into alcoholic potash and boiled under a reflux condenser for 2 h, during which the roots and the liquor turned yellow. The hydrolysate was removed, concentrated, and examined by chromatographic methods.

The chromatograms run from the hydrolysate of *S. campanulata* roots showed the presence of substances corresponding to  $\alpha$ - and  $\beta$ -glycerophosphate (the  $\beta$ -form is produced readily from its isomer by hydrolysis), inositol, serine, and possibly arginine; a small amount of material resembling choline was present on the starting line. The hydrolysate from *Trillium* roots contained material which did not move from the starting line and which gave the colour reactions for choline, phosphate, and reducing sugars;  $\alpha$ -glycerophosphate and possibly serine and arginine were also detected. These results were similar to those reported for reprecipitated nucleohistone (above). From *Vicia* roots,  $\alpha$ -glycerophosphate, some reducing sugars, and traces of amino-acids resembling serine and possibly arginine were obtained.

It seems likely, therefore, that lipoidal material was present in the roots after treatment with trichloroacetic acid and that this matter, or at least some of it, was rendered soluble in the potash but was not hydrolysed to its components. Hence it behaved similarly to the bound lipids of the calf thymus nucleohistone.

#### DISCUSSION

There have been two major obstacles to the histochemical study of the phospholipids, the first being that frequently reactions are intensified by treatment with reagents which would be expected to remove these substances, and the second that nucleohistone is an 'interfering substance', that is it stains as if it were a phospholipid. The data presented in this communication suggest that the solution to these problems lies in the possibility that true masked lipids occur which are so tightly bound that the usual lipid-solvents are unable to remove them. This binding may be so firm that boiling alkali, which hydrolyses free lecithin-like phospholipids completely, may not even dissolve these lipid-protein complexes, as was found with the sediment *C*. Moreover, not more than about 10% of the material extracted by hot pyridine from calf thymus was lipid (also see Olley and Lovorn, 1954). Thus it seems likely that hot pyridine removes non-lipid matter, possibly protein, which normally masks tightly bound phospholipid; this lipid can be visualized also by treatment with trichloroacetic acid. Hence it would appear likely that in both the nucleus and cytoplasm, truly masked lipids are present which, without special treatment, are not available for staining by virtue of their association with some other substance such as protein and nucleic acid.

Some idea of the manner in which this protection is conferred was suggested by the study of phospholipids in nucleohistone. Since nucleohistone, prepared by two different methods, contained phospholipid-like matter, perhaps in relatively high concentration, it is not surprising that nuclei and chromosomes give positive reactions for phospholipids. The binding of these lipids to the nucleohistone, however, is so strong that they may not be available to the staining reagents before the bonds linking them are broken. This would explain the increase in staining for phospholipid by nucleohistone and by nuclei after treatment with pyridine, trichloroacetic acid, and other solvents.

It has been shown that after extraction with trichloroacetic acid, when an increase in such staining was observed, lipid-like matter was present in appreciable quantity in the tissues.

In the authors' opinion it is advisable to restrict the term 'masked lipids' to those lipids which are truly 'masked', namely rendered unavailable to the staining reaction by virtue of their binding or close physical association with other substances such as protein. Lipids which cannot be resolved easily because of their colloidal state may often be visualized by the use of a more sensitive method, such as Berg's benzpyrene technique (Berg, 1951). Such lipids, in the authors' view, should be called 'finely dispersed'; they can be distinguished readily from masked lipids by their solubility.

That extraction with pyridine enhances lipid reactions has been observed in many tissues and raises the problem of whether this close association of phospholipid and protein may not be very widespread. The more recent biochemical work of Folch and Lees (1951), Folch and others (1951 a), Spiro and McKibbin (1956), Smith and others (1957), and Bruemmer and Thomas (1957), as well as the histochemical studies of Berenbaum (1954), Serra (1958), and ourselves, all suggest that frequently protoplasm may be a lipid-protein complex.

Some of the difficulties concerning the possible presence of phospholipid on chromosomes would seem to have been clarified, and defined more sharply. Mitotic chromosomes in plants and the heterochromatic segments of many interphase nuclei contain a phospholipid-like substance which is bound to protein, the whole complex being soluble in formalin (Chayen and others, 1959). It is likely that the lipid moiety would be removed from tissues by ethanol-ether or by pyridine. These solvents do not remove the staining properties of chromosomes and nuclei, however, but increase them. It is suggested that this effect is due to the increased availability of the tightly bound phospholipids of the nucleoprotein of chromosomes and interphase nuclei.

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# What is the 'Golgi Apparatus' in its Classical Site within the Neurones of Vertebrates?

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## SUMMARY

A reticulum can be seen by interference microscopy in the cytoplasm of the living neurone of vertebrates. The reticulum consists of irregular, massive bodies and thin strands. There are also well-defined spaces in the cytoplasm, in contact with the reticulum; they are usually crescentic.

The massive bodies are the objects commonly called Nissl bodies. The thin strands are the basophil threads clearly recognized by Nissl himself as constituting a part of his basophil material.

The classical 'Golgi apparatus' of the cell-body of the neurone of vertebrates consists of a deposit of silver or of osmium on the cytoplasmic inclusions mentioned in the first paragraph, but especially on the basophil strands, which have a particular affinity for silver.

At the base of the axon there are non-basophil threads, which are also blackened by the Golgi methods.

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## INTRODUCTION

ALTHOUGH the ‘Golgi apparatus’ was first recorded in the neurones of vertebrates (Golgi, 1898 *a, b*), there is still no agreement about the object in these cells which has such a remarkable affinity for silver and osmium and presents the classical Golgi pictures. A vast amount of literature on this subject has accumulated during the past 60 years, and various interpretations of the ‘appareil réticulaire interne’ of Golgi (1898*a*) have been put forth. An extensive review of these papers is not necessary, but it is intended to make reference to a few of the chief assumptions concerning this contentious object.

In 1910 Legendre considered that the picture produced by the Nissl bodies after metallic impregnation in silver had essentially the same appearance as the classical Golgi apparatus. Brambell (1923) also recognized that the Nissl substance in the neurones of vertebrates had the same distribution as the ‘Golgi apparatus’. These views have not been sufficiently examined.

Monti (1915), a pupil of Golgi, believed that the mitochondria represented in life the latter’s reticular apparatus. She based her conclusions on the resemblance between the aspect, position, quantity, and orientation of the ‘chondriome’ and the reticular apparatus in the neurones of vertebrates and arthropods. Thomas (1948, 1951, 1952) claimed that in the neurones of vertebrates the mitochondria, together with the lipid droplets, formed a scaffolding for the deposition of osmium, resulting in a netlike structure. Baker (1957*a*) also stated that ‘lipid globules and perhaps mitochondria appear to be concerned in the production of the Golgi apparatus in the neurones of vertebrates. I have also made a similar suggestion (1958).

Covell and Scott (1928) thought that the classical ‘Golgi apparatus’ was formed by the neutral red vacuoles, which were arranged in lines, and fused under the influence of fixatives. The osmium or silver was deposited on these strands thus formed.

Gatenby and Moussa have for a considerable time insisted on the existence of certain osmophil and argentophil canalicular structures (see Gatenby, 1953, 1954; Moussa, 1952, 1956; Moussa and Banhawy, 1954), which, they consider, is the classical Golgi apparatus of the neurones of vertebrates. Gatenby (1953) and Moussa (1952) and Moussa and Banhawy (1954) claim to have seen a canalicular reticulum in the living neurones of the mouse and of amphibians by phase-contrast. Adamstone and Taylor (1953) believe that they saw undulations of a dark Golgi reticulum in the living neurones of the rat by direct microscopy. The majority of the cytologists in the past have failed to observe in the living neurones either by direct or by phase-contrast microscopy any canalicular or other structure corresponding to the reticular apparatus of Golgi (Covell and Scott, 1928; Baker, 1944, 1949, 1953 *a, b*, 1955, 1957*a*; Thomas, 1948, 1951, 1952; Bourne, 1950, 1951; Gresson, 1952; Casselman and Baker, 1955; Young, 1956; Malhotra, 1957 *a, b, c*, 1958; Engström and Finean, 1958); and they have regarded the ‘Golgi apparatus’ of fixed preparations as an artifact.

Palay and Palade (1955) have described what they call an 'agranular reticulum' in the neurones of vertebrates, and tentatively suggested its homology with the 'Golgi apparatus' of other cells. This reticulum is seen in electron-micrographs of thin sections as cavities, bounded by membranes; the latter appear as closely packed, parallel profiles. Beams and his colleagues (1952), however, observed Golgi 'filaments' by electron-microscopy, identical with those seen by light microscopy (Beams, 1931). Hartmann (1953) and Mossack and Wyburn (1954) observed Golgi vesicles with lamellar membranes in electron-micrographs of vertebrate neurones.

The present investigation was undertaken with a view to determine the object on which silver or osmium is deposited to produce the Golgi apparatus in its classical site, the neurones of vertebrates.

### MATERIAL AND METHODS

It was hoped to repeat, as far as possible, the original observations of Golgi. For this reason the Purkinje cells of a bird were the first object of study. The pigeon, *Columba livia*, was selected because it is readily available. The investigation was soon extended to the neurones of the dorsal root ganglia (d.r.g. cells) of the same animal, which are much easier to examine during life. For reasons that will appear, it was decided to widen the scope of the investigation, and a certain number of observations were made on neurones from the following sources: d.r.g. cells of the mouse, anterior mesenteric and iliac ganglia of the rabbit, spinal cord of the mouse and frog (*Rana temporaria*). The main investigation centred, however, on the pigeon. The following techniques were used.

#### Phase-contrast and interference microscopy

Neurones from freshly dissected animals were teased and examined in a drop of saline (Baker, 1949). Neurones from the pigeon were studied by phase-contrast alone, the d.r.g. cells of the mouse by both phase-contrast and interference microscopy. The interference microscope has been found to be a valuable instrument for purely morphological study.

#### Trivalent dyes

Neutral red and methylene blue were used in weak solutions made in saline (a drop of 0.5% aqueous solution of the dye to 2 ml of saline) on the d.r.g. cells of the pigeon.

#### Direct osmication

Pieces of d.r.g. of the pigeon were dropped into a solution of 2% osmium tetroxide and kept at room temperature in the dark. After about 12 to 15 hours they were taken out, teased in saline, and studied by direct and phase-contrast microscopy.

### ‘Golgi’ impregnation methods

Besides the standard silver methods of Ramón y Cajal (1914), Da Faria (1920), and Aoyama (1929), Golgi’s (1898a) original method of fixation in dichromate-osmium mixture, Veratti’s (Golgi, 1900) modification of Golgi’s original method by addition of chloroplatinic acid, and Golgi’s (1908) arsenious acid / silver method were tried. Whereas the first three techniques generally produced the desired Golgi pictures, the others seldom gave any consistent results. After repeated attempts they were discarded as unsuitable for this work.

The Mann-Kopsch (Weigl, 1910) and Kolatchev (1916) methods were used: Champy fixation was adopted for the latter. The pieces of cerebellum were fixed in Mann’s fluid for 3 to 4 h, and the dorsal root ganglia and the sympathetic ganglia for 5 to 6 h. It was found that the d.r.g. cells and the sympathetic ganglion cells required about 5 to 7 days’ osmication (2% osmium tetroxide at 34° C). Shorter periods of osmication produced similar appearances in the Purkinje cells. When Mann’s fluid was used with the addition of acetic acid (Baker, 1957b), the general fixation was improved, and reduced periods of post-osmication (4 days) resulted in ‘Golgi’ pictures. Fixation in Hermann’s fluid (1889) followed by osmication (Baker, 1957b) gave quite satisfactory results in the Purkinje cells, but the fixation was not satisfactory with d.r.g. cells.

Since the above silver and osmium methods involve the use of protein coagulants in fixation, it was decided to replace such fixatives by non-coagulants, to ensure a more homogeneous appearance of the ground cytoplasm. One such technique was developed on the same lines as Baker’s (1944) method of post-osmication after formaldehyde-saline. Formaldehyde-calcium (Baker, 1944) was selected as the fixative. After fixation (24 h), the tissue was washed for about half an hour in two or three changes of distilled water, and transferred to 1% osmium tetroxide at 34° C. Osmication for 4 days was found to be sufficient to produce the ‘Golgi’ pictures. Sections were cut by Peterfi’s methyl benzoate / collodion method (Lee, 1937).

Altmann’s (1894) was also selected as a fixative for this purpose, because it is known to be excellent for cytoplasmic studies. Osmication after Altmann gave a good view of the Golgi apparatus against a smooth background. The following are the details of the procedure adopted for osmication after Altmann.

1. Fix ganglia for 24 h.
2. Wash in running water for 24 h.
3. Transfer to 2% osmium tetroxide at 34° C for 6 or 7 days.
4. Wash in running water overnight.
5. Dehydrate, pass through toluene, and embed in paraffin. Cut sections at 6  $\mu$ .
6. Mount in Canada balsam.

Osmicated sections were sometimes bleached in turpentine, sometimes in

Veratti's mixture of potassium permanganate and sulphuric acid (Lee, 1937). The latter acts readily, and the sections are decolourized within a quarter of an hour. Turpentine, sodium iodate, and hydrogen peroxide are slow and not recommended for this purpose. If the fixative contained mercuric chloride, sections were treated with  $\frac{1}{2}\%$  iodine in 70% alcohol and sodium thiosulphate. They were then stained in basic dyes (see below). For staining in cresyl violet (see below) the sections were coated in collodion.

For desilvering (Adamstone, 1952), the sections were brought down to 0% alcohol, bleached in iodine-alcohol, washed thoroughly in changes of 0% alcohol, and brought to distilled water. They were transferred to 0.75% silver nitrate to remove free iodine, and directly into 5% sodium thiosulphate to remove un-reduced silver. After washing in running water the sections were ready for staining.

#### *Mitochondrial techniques*

The three fixatives that were found best for studies of mitochondria were Helly (1903), Altmann, and a new modification of Altmann (Baker and Luke, 1958), in which ammonium dichromate is substituted for potassium dichromate ('NH<sub>4</sub>-Altmann'). Material fixed overnight in Helly was postchromed in a saturated solution of potassium dichromate at 37° for 24 to 30 h. The staining methods of Metzner (1928) and Hirschler (1927) were used. Sections of tissue fixed in Helly were also stained in acid fuchsin and differentiated in toluidine blue (Bensley, from Cowdry, 1913). Such sections were treated with 0.5% aqueous solutions of potassium dichromate for 30 sec before staining, as recommended by the author. Mitochondria were also stained in sections that had been deosmicated or desilvered by the methods given above.

Collodion sections of Altmann and NH<sub>4</sub>-Altmann, 10-20  $\mu$  thick, were examined unstained by phase-contrast or interference microscopy.

#### *Colouring agents for lipids*

Neurones fixed in formaldehyde-calcium (Baker, 1944) and Flemming with acetic acid were coloured in Sudan black (Baker, 1949, 1956). The acid aematein test of Baker (1946) was used for the detection of phospholipids.

#### *Basic dyes for Nissl substance*

Tissue fixed in various ways was stained in basic fuchsin (0.5% aqueous), toluidine blue (0.5-1%, Young, 1932), thionine (0.2%), gallocyanine (Einarson, 1933), pyronin / methyl green (Jordan and Baker, 1955), and cresyl violet (Fernstrom, 1958). Fixation in Mann's fluid with acetic acid (Baker, 1957b) followed by staining in cresyl violet was excellent for showing basophil material. Pyronin / methyl green after Zenker and Mann's with acetic acid also gave good results.

Staining with basic fuchsin is very simple, besides being extremely good, especially after fixation in Mann's fluid with acetic acid. Sections were brought to water (through iodine-alcohol and sodium thiosulphate, if necessary). They

were stained in a 0.5% aqueous solution of basic fuchsine. After rinsing in distilled water, they were passed through alcohols rather quickly, with differentiation in 70% or 90% alcohol if desired, cleared in xylene, and mounted in balsam.

Sections of material fixed in mixtures with osmium tetroxide were bleached in potassium permanganate and oxalic acid before staining. The treatment of post-osmicated and silvered material before staining is mentioned above (p. 343).

Extraction of the basophil material was done by treatment of sectioned material with ribonuclease (Bradbury, 1956) or 5% aqueous solution of trichloroacetic acid (Pearse, 1954).

## RESULTS

### *Purkinje cells and dorsal root ganglion cells of the pigeon*

*General remarks.* These two kinds of cells are so closely similar in their cytoplasmic inclusions that a single account of them will suffice. On the whole the d.r.g. cells are easier to study, because they are larger. Certain observations were made on these cells only, but there is no reason to suppose that Purkinje cells different. It will not be necessary to mention in every case to which kind of cell a particular observation was made.

There is a good deal of size variation in the d.r.g. cells. They vary from about 15 to 50 $\mu$  in diameter. There does not seem to be so much variation in the size of the Purkinje cells. Most of them measure about 20 $\mu$  in width. The nucleus, in both kinds of cells, usually contains one large nucleolus (sometimes two). The nucleus is almost centrally placed, and is generally spherical but sometimes ovoid.

*Living cells.* It is rather difficult to get fresh Purkinje cells in really good condition for study in the living state, because even a slight pressure on the coverglass is damaging to the cells, and vacuolation sets in rather quickly. The task is much easier if d.r.g. cells are used for this purpose. In this case, however, since these ganglia are very compact structures, it is essential that the tissue be extremely finely teased and the coverslip pressed to flatten the cells. The tissue was teased and mounted in saline solution (Baker, 1944).

In freshly mounted neurones examined under phase-contrast, only one type of cytoplasmic inclusions can be seen with clarity. These are numerous spherical and subspherical globules of high contrast, scattered at random throughout the cytoplasm except at its extreme periphery, where there are a few. They are of varying sizes, but the biggest measure less than 1 $\mu$  in diameter in the Purkinje cells. The smallest tend to be arranged in rows. The globules develop a higher contrast when they escape from disintegrating cells into the saline medium. The globules appear colourless by direct microscop-

Mitochondria are extremely difficult to see in the freshly teased neurones even by phase-contrast. After some time, when the cells have been flattened by the coverglass, it becomes possible to see small fine threadlike structures suggestive of mitochondria.

Studies under phase-contrast failed to reveal anything else significant in the cytoplasm of living, unstained neurones of the pigeon. I have seen some canalicular spaces in the living d.r.g. cells of the mouse and these will be mentioned below (p. 355).

When the cells are vitally dyed with neutral red or methylene blue, the colour is taken up by spherical cytoplasmic inclusions, which are thereby made evident. These spheres correspond in size and distribution with the highly refractile globules seen by phase-contrast, and there is no reason to doubt their identity.

*Osmium preparations of whole cells.* In d.r.g. cells osmicated for 12 to 15 h and examined in saline by direct microscopy, greyish globular bodies are seen. They appear to be similar to the ones observed in the living unstained neurones by phase-contrast. Under phase-contrast, one sees with some clarity in these cells, besides the globular bodies, which now appear very dark, extremely fine, long, threadlike bodies, which look superficially like mitochondria. Some of them are in association with the edges of crescentic spaces (fig. 1) similar to those seen in the living, unstained d.r.g. cells of the mouse (p. 355). The threads form a rim on one side of the crescentic spaces. This suggests that these threadlike organelles are not mitochondria. These structures could not be seen by direct microscopy.

Neurones soon become opaque in osmium tetroxide solution and it was not found possible to make further observations by this method.

*Preparations for lipid inclusions.* Colouring with Sudan black after fixation in formaldehyde-calcium or Flemming with acetic acid reveals the existence of small lipid globules of varying sizes (fig. 2, A). These appear to be the same structures as the globular bodies described in the above paragraphs. They are mostly homogeneous, but a few of them in the d.r.g. cells may show a sudanophil cortex and sudanophobe internum in material fixed in formaldehyde-calcium. The lipid globules are, however, invariably homogeneous after colouring in acid haematein.

Sudan black and acid haematein preparations also sometimes reveal clearly canalicular spaces, generally crescentic, dispersed in the cytoplasm. There does not seem to be any lipid material in association with these spaces.

*Mitochondrial preparations.* Of all the mitochondrial techniques listed earlier in this paper (p. 343), fixation in Helly, followed by post-chroming and staining by the methods of Metzner and Hirschler, has given the best results with

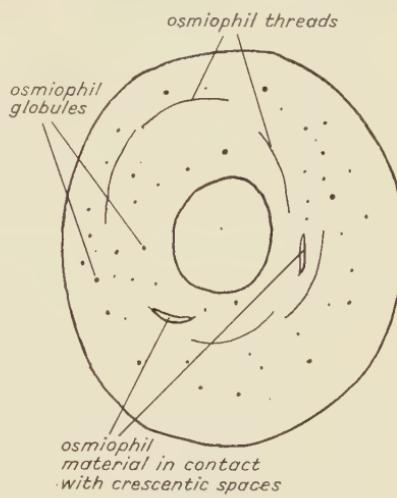


FIG. 1. D.r.g. cell (pigeon), osmicated for 12 h at room temperature and studied whole under phase-contrast.

the d.r.g. cells. Fixation in  $\text{NH}_4$ -Altmann followed by staining by the method of Metzner was quite satisfactory for showing mitochondria in both kinds of cells. In these preparations mitochondria appear as fine, curved threads dispersed in the cytoplasm. The longest of these do not exceed  $4\ \mu$  in the d.r.g. cells. They appear to be shorter in the Purkinje cells. It is sometimes possible to resolve the filaments into granules lying in rows. In these preparations granular bodies corresponding to the lipid globules are also seen scattered throughout the cytoplasm (fig. 2, B).

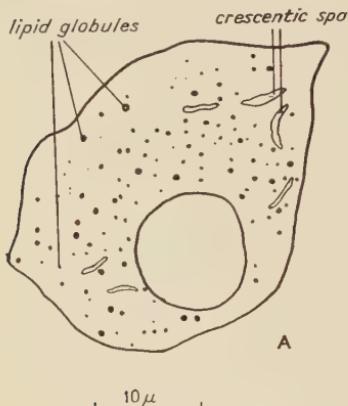


FIG. 2.

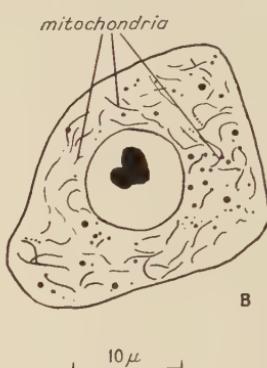


FIG. 2.

FIG. 2. A, d.r.g. cell (pigeon); formaldehyde-calcium / Sudan black, showing the lipid globules. There is no sudanophil material in contact with the canalicular (crescentic) spaces. B, d.r.g. cell (pigeon), Helly / post-chromed / Metzner, showing mitochondria. Lipid globules are also stained by acid fuchsin.

FIG. 3. Purkinje cell (pigeon), prepared by Golgi's arsenious acid / silver nitrate method, and stained in basic fuchsin after desilvering to show basiphil (Nissl) substance. Note crescentic spaces in contact with the basiphil substance.

There is some evidence that the mitochondria are blackened by osmium. In neurones processed by the technique of Baker (1957b) after fixation in Mann's fluid with acetic acid or in Hermann's fluid, small rod-like structures are seen, resembling the mitochondria of Helly and  $\text{NH}_4$ -Altmann preparations.

In acid haematein preparations also, one comes across feebly coloured thread-like bodies, which can be just seen against the yellowish background of the cytoplasm.

*Nissl preparations.* The basiphil material of the neurones is seen in two forms. One of these consists of large, diffused lumps of varying sizes, dispersed throughout the cytoplasm. These correspond to what are commonly described as Nissl bodies. The other is represented by basiphil strands which connect the Nissl bodies with one another (figs. 3; 4, E). Some of these strands are extremely fine. They are sometimes closely applied against the Nissl bodies and the latter, in such cases, appear to be sharply demarcated from the cytoplasm, on the side on which the strand is applied. These strands are also seen associated in the form of loops with the Nissl bodies. In fact, the



FIG. 3.

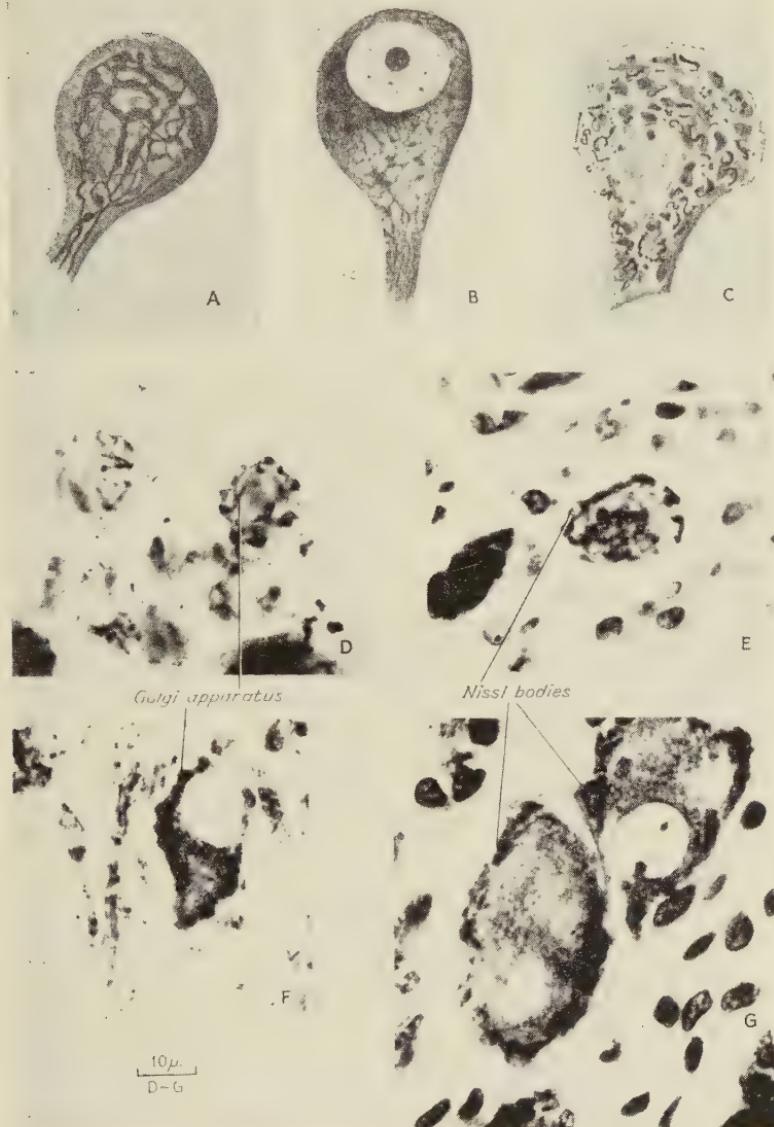


FIG. 4. A, Purkinje cell from barn-owl (*Strix flammea*) showing 'appareil réticulaire interne' of Golgi; reproduced from Golgi (1898a). B, Purkinje cell from rabbit, showing basiphil reticulum; reproduced from Dolley (1913). C, d.r.g. cell from rat, prepared by Mann-Kopsch method, showing black 'Golgi filaments' and greyish 'Nissl bodies'; reproduced from Beams (1931), by courtesy of Professor H. W. Beams. D, Purkinje cell from pigeon, showing Golgi apparatus made by Aoyama method. E, Purkinje cell from pigeon; Mann's with acetic acid and cresyl violet, showing basiphil material. F, sympathetic ganglion cell from rabbit; Mann-Kopsch, showing massive blackened bodies at the periphery of the cell. G, sympathetic ganglion cell from rabbit; Mann's with acetic acid and cresyl violet. Note massive Nissl bodies at the periphery of cell.

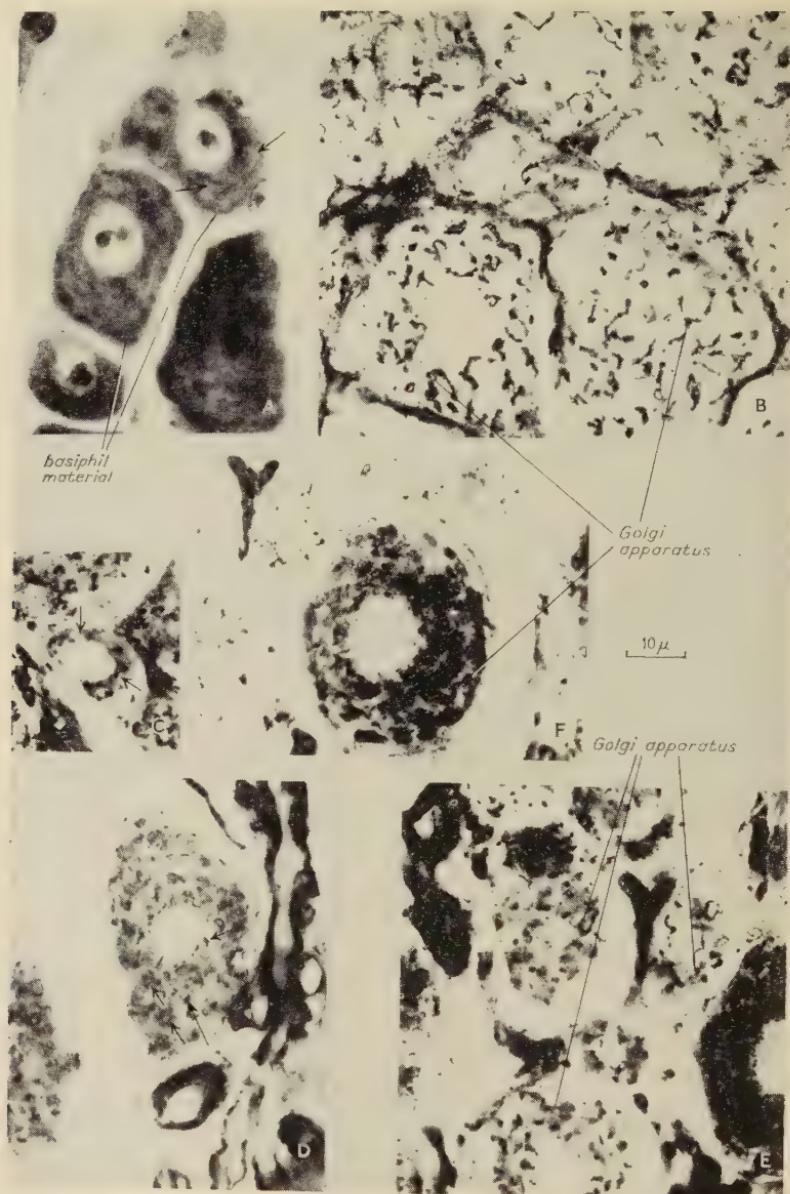


FIG. 5. A, d.r.g. cells from pigeon; Helly / post-chromed / acid fuchsin and toluidine blue. Basophil material in focus. B, d.r.g. cells from pigeon; showing Golgi apparatus by the method of Aoyama. C, D, d.r.g. cells from pigeon; Mann-Kopsch; arrows indicate crescentic spaces with or without blackened edge. Small rod-like bodies are also seen in D. E, d.r.g. cells from pigeon, Mann-Kopsch, showing filamentous Golgi apparatus corresponding to that seen by Aoyama method (fig. 5, B). F, d.r.g. cell from pigeon; Mann-Kopsch showing massive form of the Golgi apparatus after 6 days of osmication.

asiphil strands seem to exist in the form of a three-dimensional reticulum, associated with the Nissl bodies. Dispersed in this reticulum one often sees crescentic or rounded spaces either within the Nissl bodies or in association with the basiphil strands (fig. 5, A).

In suitable sections of material fixed in Helly (with post-chroming), stained with acid fuchsin, and differentiated in toluidine blue, mitochondria and basiphil reticulum can sometimes be seen side by side in the same cell.

When sections have been extracted with ribonuclease or trichloroacetic acid, it is no longer possible to colour either the Nissl bodies (in the narrower sense) or the basiphil strands with basic dyes. Moreover, a bleb of fresh egg albumin, which does not contain any nucleoprotein, was treated exactly as the manner Nissl preparations were made and it was found that the egg albumin had hardly been stained in the time given for staining Nissl substance.

A note on terminology is necessary. The Nissl complex (Nissl bodies and basiphil strands) consists of endoplasmic reticulum, with ‘small granules’ of Palade (1955a) scattered on its surfaces (Palay and Palade, 1955; Palay, 1956). Basic dyes presumably react only with the ribonucleoprotein of the scattered granules. Nevertheless, the terms Nissl bodies and basiphil strands are used throughout this paper to mean the whole of these objects, not their basiphil component only.

*Golgi preparations.* The term ‘Golgi apparatus’ has been used throughout this paper to denote the black or grey network seen in silver or osmium preparations made by any of the classical ‘Golgi’ techniques.

Small dark granules are seen in the cytoplasm in Golgi preparations (fig. 5, D, E). Sometimes they show a dark cortex and colourless medulla. These are presumably the lipid globules. Small rod-like bodies are also sometimes seen in osmicated Golgi preparations. These are presumably mitochondria.

Golgi preparations of the Purkinje cells made by the Mann-Kopsch method (4 days’ osmication) show long, curved or straight filaments dispersed in the cytoplasm. Similar structures are seen in these preparations of the d.r.g. cells (fig. 5, E) especially near the periphery of the sections (6 days’ osmication). Some of these are seen to form a rim partly encircling clear canalicular spaces. The canal may be as long as the filament, or shorter. Some characteristic structures are seen in the d.r.g. cells, especially in the neurones lying in the more central part of the section. These are mostly crescentic (rarely rounded), sharply demarcated structures. The edge of these shows great affinity for osmium, and the latter is generally deposited on one side only, rarely on both sides (fig. 5, C, D). When it is on one side only, these structures show a superficial resemblance to the dictyosomes of neurones of invertebrates (Dornesco and Busnitz, 1935; Pollister, 1939). These are identical with those seen when osmicated whole cells are examined by phase-contrast microscopy (p. 345). In these cells one also sees a few canalicular spaces, which show a deposit of osmium just beginning to be formed on the edge, and others with no such deposit (fig. 5, C, D, E).

In Mann-Kopsch preparations osmicated for slightly longer periods (7 days), many of the d.r.g. cells show, besides the structures described, massive bodies dispersed throughout the cytoplasm. All these structures seem to be connected into one net-like structure (fig. 5, F). Some of the dark, massive, irregular bodies show blackened filaments closely applied to them. Rounded, elongated swellings, hollowed out internally, are seen dispersed in this net-like system. In the Purkinje cells prepared by the same technique (4 days osmication) blackened filaments predominate, and form a meshwork, in which dark swellings are rarely seen. The picture seen in both types of neurones resembles the classical Golgi apparatus.

All the structures described in the d.r.g. cells in Mann-Kopsch preparations can be clearly seen in neurones fixed in Mann's fluid with acetic acid and post-osmicated for 4 days. Massive bodies are often seen, with crescentic or rounded canalicular spaces inside. As one focuses up and down, one can sometimes see that a canalicular space lies up against a Nissl body, which may, indeed, enclose it.

In Aoyama preparations, one generally sees long filaments forming a meshwork (fig. 5, B). Crescentic canalicular spaces with argentophil rim are sometimes present; but they are distorted and difficult to see against the whitish background of these preparations. These preparations show a few varicosities interposed in the filamentous Golgi net (fig. 4, D). They are either homogeneously dark or show a hollow interior. But massive bodies of the type seen in osmium preparations are lacking in Aoyama preparations. The filamentous Golgi net seen in Aoyama preparations therefore contrasts with that observed in osmium preparations, in which bulky, irregular structures are interposed among the filaments. Some of the Golgi filaments may be as long as  $10\mu$ , i.e. more than twice the length of the mitochondria.

I have failed to get any consistent results with the silvering methods used by Golgi himself. I have, however, come across the structures illustrated in fig. 6 in a Purkinje cell prepared by Golgi's dichromate-osmium method. This figure shows a massive, pale orange body (presumably a Nissl body) in association with a long greyish thread and clear crescentic spaces, resembling those seen in osmium preparations of the d.r.g. cells.

FIG. 6. From a Purkinje cell from pigeon prepared by Golgi's dichromate-osmium method showing the association of canalicular spaces, Golgi thread, and a massive body (presumably a Nissl body).

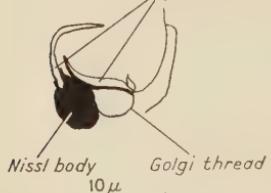


FIG. 6. From a Purkinje cell from pigeon prepared by Golgi's dichromate-osmium method showing the association of canalicular spaces, Golgi thread, and a massive body (presumably a Nissl body).

In the Purkinje cells the Golgi filaments are seen extending into the axons, as recently described by Gatenby and others (1949, 1953). This is contrary to the prevailing opinion (Pappenheimer, 1911; Penfield, 1920; Beams and others, 1952; Singer, 1954). Golgi (1898a) himself does not seem to have made any reference to the internal reticular apparatus in the cell processes, but his figure (reproduced here in fig. 4, A) does indicate the continuation of the reticular apparatus into a cell process, which probably a dendrite.

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In sections of the dorsal root ganglion processed by Mann-Kopsch or Baker's (1957b) post-osmication method after fixation in Mann's fluid with citric acid, Golgi pictures confined to one half of the cell are frequently seen. The cause is presumably uneven penetration of osmium. Such cells are of common occurrence near the periphery of the sections. If such preparations are studied after staining in basic fuchsin or in pyronine / methyl green, the Golgi apparatus is seen in one half of the cell and the basophil material in the other half. Careful study of such neurones reveals that the Golgi filaments in one part of the cell can sometimes be followed into basophil strands in the other half. Moreover, in some of these cells the large dark masses look identical with the basophil masses (Nissl bodies) in the other half. But in those cells which showed almost complete impregnation with osmium, neither the Nissl bodies nor the basophil strands could be demonstrated by the use of basic dyes. In silver preparations stained in basic fuchsin or pyronine / methyl green, the Nissl bodies are generally dispersed in the cytoplasm amongst the Golgi filaments, but the basophil strands cannot generally be observed in such cells. The silvered Golgi filaments could in a few instances be seen to be associated with the Nissl bodies.

If osmication or silvering happens to be scanty in any particular cell, the pattern of the Golgi picture overlaps the underlying pattern of the basophil material, and sometimes grains of osmium or silver can be seen along the length of the basophil strands.

Silver preparations can easily be stained after toning in gold chloride. With osmium preparations, staining could only be applied to those neurones in which the ground cytoplasm had not turned dark, because any attempt to reach these preparations tended to remove osmium from the cytoplasmic inclusions.

*Unstained sections studied by phase-contrast and interference microscopy.* Neurones fixed in Golgi fixatives but not osmicated nor silvered (or else reached after osmication or silvering) were examined in sections by phase-contrast microscopy. The intention was to determine whether any reticulate other structure, corresponding to the Golgi apparatus, could be seen. Sometimes long filaments were seen, often associated with large masses, resembling the Nissl bodies of routine preparations. These filaments are too long to be mitochondria. Since most of the Golgi fixatives contain coagulants and proteins, it was thought that these filaments might be coagulated cytoplasmic proteins. However, the evidence does not support this view. Structures resembling them could be seen in neurones fixed with a non-coagulant fixative (Golgi's dichromate-osmium) and stained in basic fuchsin. Moreover, when sections have been treated with ribonuclease, these bodies can no longer be stained with basic dyes.

The constant appearance in fixed preparations of characteristic canalicular spaces led to the study of unstained sections by interference microscopy. These spaces were so sharply demarcated in Golgi preparations that it was difficult to believe that they were mere cracks caused by the shrinkage of the

cytoplasm, with deposition of osmium or silver on the surface of the cracks. But for some time there was no other satisfactory explanation for them. Since they were visible by phase-contrast in whole cells that had been put directly into osmium tetroxide solution, but could not be seen in unstained, living fresh neurones, it was assumed that they were the result of the action of fixatives. They were also seen in the tissue that had been embedded in gelatin.

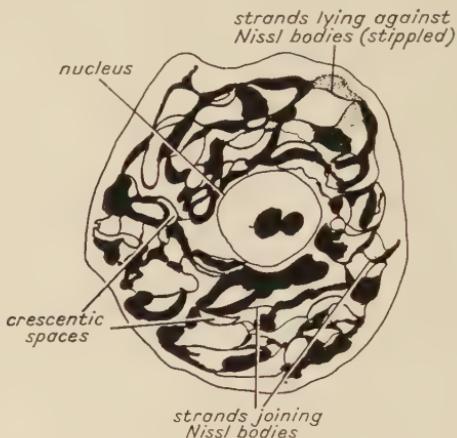


FIG. 7. D.r.g. cell from pigeon fixed in  $\text{NH}_4$ -Altmann and cut by embedding in collodion; studied unstained by interference microscopy, showing a reticulum consisting of large, irregular, massive bodies (presumably ‘Nissl bodies’) and thread-like strands joining Nissl bodies. Sometimes the strands are seen lying against the Nissl bodies (stippled to show this relation). Crescentic spaces are also seen both in contact with the Nissl bodies and the strands.

Later, when these canalicular spaces were seen in osmium preparations within bodies resembling Nissl masses, it seemed likely that they were produced by the contraction of the Nissl bodies away from the ground cytoplasm or the retraction of the latter from the Nissl bodies, during the process of fixation. It was thought probable that if the tissue were fixed in a fluid which would preserve the cell inclusions in their living state, and sections were cut after embedding in collodion, these spaces would probably not be seen in the finished sections, unless they existed in life. Material was therefore fixed in Altmann and  $\text{NH}_4$ -Altmann, and embedded in collodion. By phase-contrast microscopy it was found that the canalicular spaces were seen even more clearly than before. Under the interference microscope, it was seen that these spaces were associated with a system in the cytoplasm which could not be made discernible in the living neurones by phase-contrast. This system is formed by two structures which seem to be closely associated with one another. It consists of large, irregular, ill-defined bodies, which resemble, in all details, the Nissl bodies of routine fixed preparations. These were seen to be connected with one another by strands of similar material (fig. 7). Some of these strands are extremely fine. Careful study of suitable sections reveals that these two structures constitute a reticulate system in these neurones.

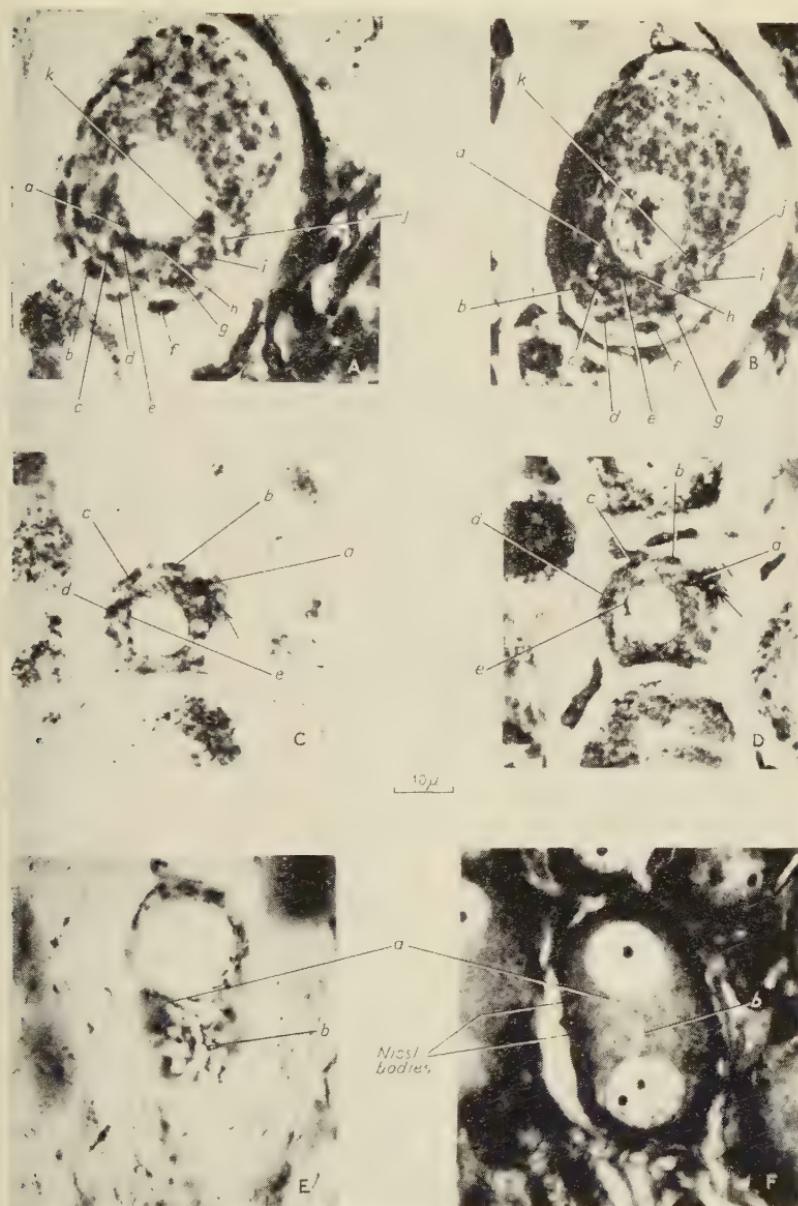


FIG. 8. All these photomicrographs have been taken to show that the Golgi pictures can be produced in the same cell by staining in basic dyes after bleaching. A, d.r.g. cell from pigeon; Ann-Kopsch. B, same cell as in A, stained in basic fuchsin after bleaching. Compare *a* and *a*, *b* and *b*, and so on in the two figures. C, d.r.g. cell from mouse; osmicated after maldehyde-calcium. D, same as in C, stained in cresyl violet after bleaching. Compare *a* and *a*, *b* and *b*, and so on in the two figures. Arrow indicates a space in association with a massive body in both C and D. E, anterior mesenteric ganglion cell from rabbit, Da Fano. F, same cell as in E, stained in basic fuchsin after bleaching. Compare *a* and *a* and *b* and *b*. Nissl bodies are seen at the periphery of the cell.

*Golgi preparations dyed after bleaching.* The filamentous form of the Golgi apparatus, which is particularly well seen in silver preparations, makes one inclined to believe that an excess of silver or osmium was deposited on the mitochondria and joined them up into a network. I have repeatedly tried to reproduce the Golgi appearances by one of the standard mitochondrial methods after removing silver or osmium, but always failed. I have also failed to colour with Sudan black or Sudan IV any part of the Golgi apparatus in paraffin or gelatine sections, either before or after bleaching.

When it was revealed by interference microscopy that the canalicular spaces

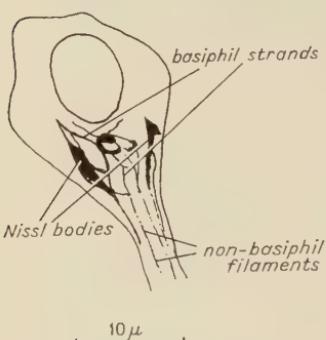


FIG. 9. Purkinje cell from pigeon; fixed in Hermann (followed by post-osmication) and stained in basic fuchsin after bleaching; showing non-basophil extension into the axon of the basophil complex.

indeed produced conclusive evidence in favour of the hypothesis that the Golgi apparatus of the neurones of vertebrates was nothing else than a deposit of osmium or silver on the basophil material, the latter forming a scaffold for Golgi impregnation.

The results of these experiments are shown by the photomicrographs. Fig. 8, A shows the Golgi apparatus in a d.r.g. cell of the pigeon in a Manz-Kopsch preparation. Fig. 8, B shows the same cell stained in basic fuchsin after the osmium had been removed. In such cells the Golgi apparatus looks like the basophil material of its cells that it can scarcely be doubted that the structure is being revealed by two different methods.

It is instructive to bleach a Golgi preparation that shows the apparatus extending into the axon, and then to stain it with a basic dye. It is thus observed that the strands which extend into the axon are not coloured by the dye. In this respect they differ from the rest of the Golgi apparatus. Nevertheless, careful examination reveals the presence of these unstained extensions in the form of narrow threads, parallel to the axon (fig. 9). These seem to be some small spaces associated with these non-basophil threads, but it is very difficult to be certain about this.

Neurones impregnated with silver are not very suitable for bleaching experiments

described as the 'Golgi apparatus' by Gatten and others, were associated with the basophil strands and the Nissl bodies, experiments were devised to determine whether the basophil material could be demonstrated after Golgi methods had been applied. There were only two ways in which such experiments could be performed. The first was to try to demonstrate the basophil material in the neurones while the osmium or silver was intact. If this had succeeded, it would have proved that the basophil material and the Golgi apparatus were independent of each other. When it was found that this could not be done, the only alternative experiment was to see what was coloured by basic dyes after osmium or silver had been removed from the sections. These experiments

evidence in favour of the hypothesis that the Golgi apparatus was nothing else than a deposit of osmium or silver on the basophil material, the latter forming a scaffold for Golgi impregnation.

ments. When the deposit of silver is removed, the strand has a somewhat different appearance, and it is not easy to be certain which strand is which. If silver darkened the massive Nissl bodies, as osmium does, it would have been easier to correlate the stained with the silvered preparation. Moreover, no one uses the fixatives of Aoyama or Da Fano for the demonstration of basiphil material, because they do not leave the tissue so readily stainable as do fixatives containing mercuric chloride. Beyond this, they are coagulant-fixatives that distort the ground cytoplasm in coagulating it.

Silvering or osmication of the basiphil strands and the Nissl bodies masks completely or partially the canalicular spaces enclosed in the basiphil reticulum. It is not uncommon, however, to find varicosities in the Golgi apparatus which are due to silver or osmium covering these spaces. This is proved when silver or osmium is removed and the real structure is made discernible by staining in a basic dye. So in a way it can be stated that the ‘wall’ of the canalicular spaces is argentophil and osmiophil, but not sudanophil.

#### *Neurones of mammals and other vertebrates*

*General remarks.* When it was found in the neurones of the pigeon that there was close relationship in the distribution of the basiphil material and of the Golgi apparatus, it was considered desirable to make a wide survey of the neurones of vertebrates. It was hoped to come across cells with a characteristic disposition of the basiphil material and then to find out the distribution of the Golgi apparatus in the same kinds of cells.

*Neurones of the mouse.* The d.r.g. cells of the mouse were selected for the study of fresh tissue by interference and phase-contrast microscopy. These cells are much easier to examine in the living condition than are those of the pigeon, because in the mouse the ganglia are smaller and easier to tease finely. Under phase-contrast, the most obvious inclusions are dark globules of various sizes (almost certainly lipid droplets). In addition one sees in the cytoplasm crescentic spaces of low contrast. These spaces have remarkably smooth and sharply defined limits.

Under the interference microscope these crescentic spaces are not so sharply demarcated, but they are seen to be associated with other bodies that are not clearly visible by phase-contrast (presumably because their boundaries are not sufficiently sharp to cause much diffraction of light). These bodies, which are of irregular shape and varying size, are dispersed throughout the cytoplasm. They look like Nissl bodies. Careful study reveals that they are connected with one another by fine strands, forming a reticulate system. The crescentic spaces mentioned above are associated both with the fine strands and also with the irregular bodies. It is, therefore, possible to visualize in the living neurones a reticulate element consisting of (i) irregular, massive, structures (presumably Nissl bodies), connected with one another by (ii) strands, which are sometimes extremely thin, and (iii) crescentic spaces disposed along the course of this reticulum (see fig. 12, p. 359).

Under the interference microscope it has not been possible to see any

minute structural details in the irregular bodies, which will hereafter be called Nissl bodies. Indeed, the numerous refractile globules, present in the cytoplasm of the living neurones, obscure a clear view of the Nissl bodies. The latter show almost the same interference colour as the strands connecting them; the colour suggests a higher refractive index than that of the ground cytoplasm. Since the strands are much thinner than the Nissl bodies, they must have a higher refractive index than the Nissl bodies. The strands do not seem to be perfectly smooth. They show a roughness of contour that might be due to the presence of minute granules in or on them, but it is difficult to be absolutely certain about this.

The basophil material of the d.r.g. cells of the mouse shows in fixed preparations essentially the same pattern as in the d.r.g. cells of the pigeon. The Nissl bodies are generally smaller than in the neurones of the pigeon. In correspondence with the distribution of the basophil material, the Golgi apparatus is also dispersed throughout the cytoplasm.

Golgi preparations were made by fixation in formaldehyde-calcium (Altmann), and with post-osmication, and also by Aoyama's method. The osmicated preparations were subjected to the same deosmication experiments as the neurones of the pigeon; they were subsequently studied after staining in cresyl violet according to the technique of Fernstrom (p. 343). The results of these experiments are indicated in figs. 8, c, D; 10, A, B. In fig. 8, c a clear elongate space is seen associated with a black mass at 2 o'clock. After bleaching and then staining in cresyl violet the same space is seen in association with what is now proved to be Nissl body.

The spinal cord was another source of neurones of the mouse for the investigation. In some of the ventral horn cells the basophil complement was seen to be concentrated round the nucleus in some cells and to extend in the form of a cone into the dendrites. When Golgi preparations were made by the method of Aoyama, the Golgi apparatus was found to have a strikingly similar disposition.

*Neurones of the rabbit.* The sympathetic neurones of the rabbit provide another example of correspondence between basophil material and Golgi apparatus. In these cells the massive Nissl bodies are generally confined to the periphery of the cell (fig. 4, G), as described by Eve (1896), Carpenter and Conel (1914), and Kuntz (1928). These basophil masses are connected by strands of similar material which are dispersed throughout the cytoplasm. Elongate, canalicular spaces are sometimes associated with the strands and with the Nissl bodies. These spaces are generally longer and thinner than in the d.r.g. cells of the pigeon.

If the corresponding neurones are studied in silver preparations, it is found that the Golgi apparatus, which is mostly made up of filaments (fig. 8, E), is confined to the region of what Casselman and Baker (1955) call the ‘endoplasm’. In osmium preparations also the Golgi apparatus is mostly filamentous in the ‘endoplasm’, and this corresponds with the lack of massive Nissl bodies in this region. But large dark greyish bodies are also seen at the periphery.

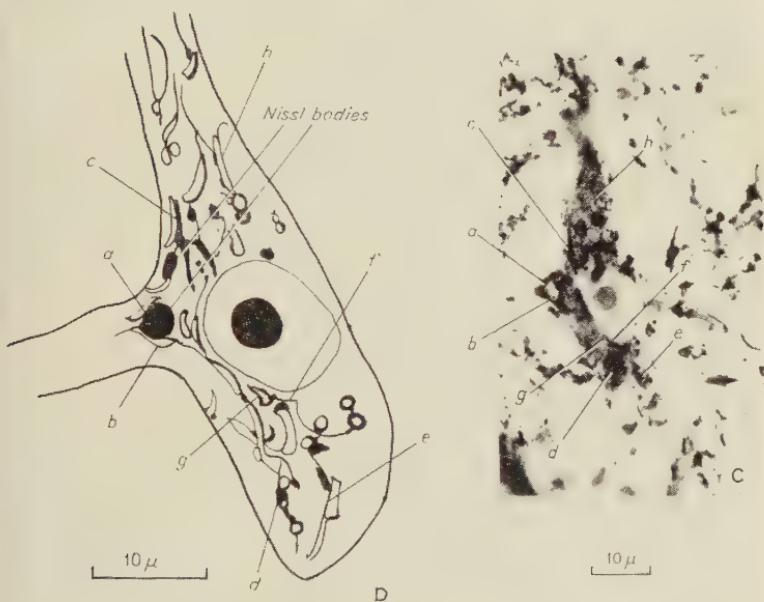
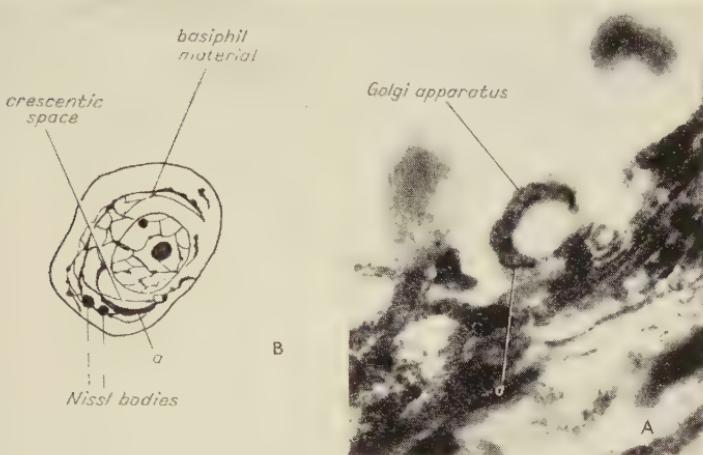


FIG. 10. These figures have been made to show that the Golgi pictures can be reproduced in the same cell by staining in basic dyes after bleaching. A, d.r.g. cell from mouse; Altmann followed by post-osmication; showing Golgi apparatus round the nucleus. B, same cell as in stained in cresyl violet after bleaching. Compare with A. C, spinal cord neurone from g; Aoyama; slightly tinged with basic fuchsin. D, same cell as in C; stained in cresyl violet after bleaching. A comparison of C with D would show that some of the swellings in C are due to deposition of silver on canalicular spaces, as at a and b.

the cell in the ‘ectoplasm’ of Casselman and Baker (1955), as seen in fig. 4, F. As was mentioned earlier in the account of the neurones of the pigeon, the filamentous part of the Golgi apparatus has a greater affinity for silver and osmium than the large, bulbous swellings which are usually later incorporated into the Golgi apparatus. In the sympathetic neurones of the rabbit the peripheral swellings begin to darken after the tissue has been in osmium tetroxide.

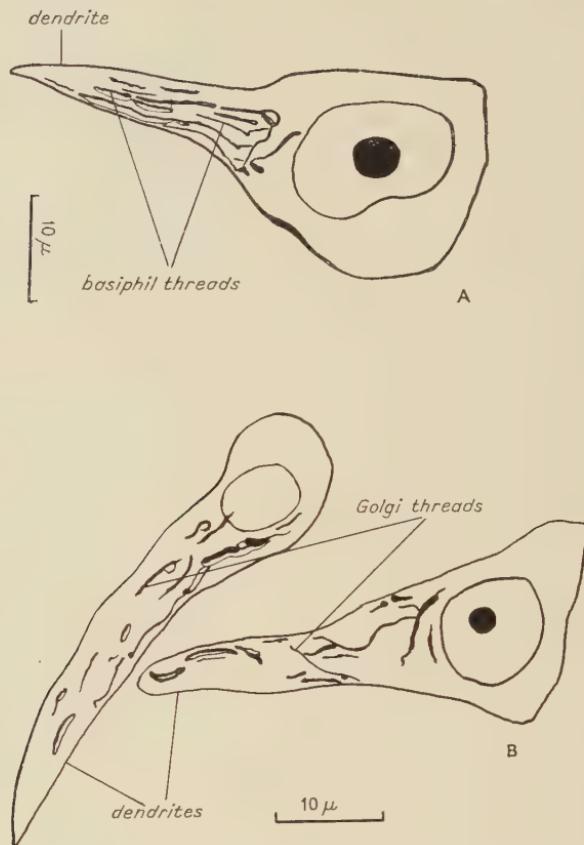


FIG. 11. Spinal cord neurones from frog, showing basophil material in dendrite (A, Helly / basic fuchsine) and similar distribution of the Golgi apparatus in the cell processes (B, Aoyama).

solution for about 4 days at 34° C (after fixation in Mann's fluid). One therefore often sees black Golgi filaments in the ‘endoplasm’ and large dark greyish bodies in the ‘ectoplasm’ (fig. 4, F). Often the Golgi filaments can be seen passing from one zone into the other. In suitable preparations the blackened filaments are seen to form a sort of ‘wall’ to the canalicular spaces.

Desilvering experiments were performed on Da Fano preparations of the anterior mesenteric ganglion cells. As was pointed out, silver preparations are not very suitable for this purpose. Nevertheless, there is a general similarity

between the arrangement of the Golgi apparatus and of the basophil material seen in the same cell after desilvering and then staining (fig. 8, E, F).

*Neurones of the frog.* Another instance of a peculiar distribution of the basophil material is met with in the spinal cord of the frog. In some of the ventral horn cells the basophil substance extends into the cell processes in the form of long strands placed along the length of the dendrites (fig. 11, A). Silver

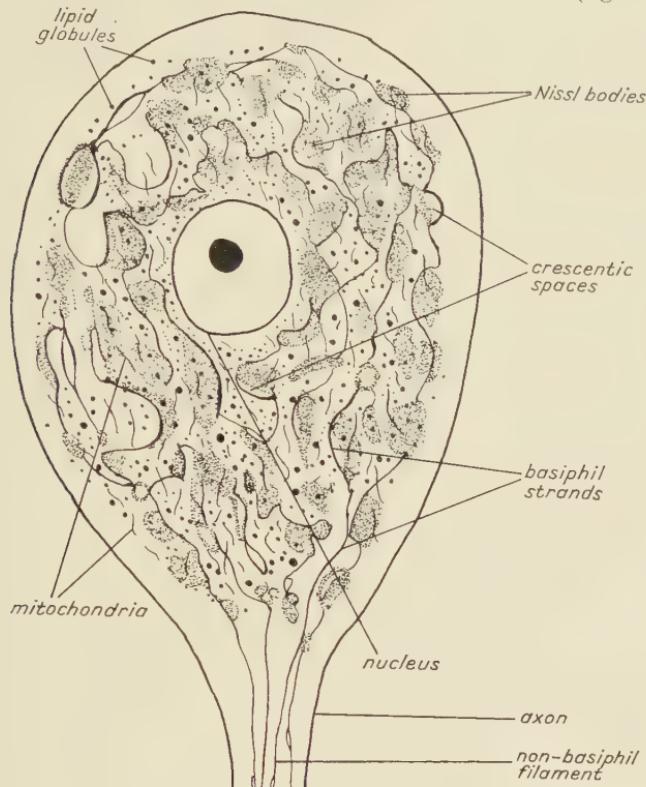


FIG. 12. Diagrammatic representation of neurone of vertebrates showing the arrangement of cytoplasmic inclusions in life. Nissl bodies have been stippled to illustrate their association with basophil strands. Mitochondria not shown in the axon.

preparations also revealed similar Golgi filaments in the cell processes (fig. 11, B). Elongated spaces are seen in association with the basophil strands and also with the Golgi filaments (fig. 11, A, B).

The results of desilvering experiments and subsequent staining by a basic dye are illustrated in fig. 10, C, D. Comparison of the two figures shows that some of the canalicular spaces seen after desilvering and then staining are covered by silver and these spaces show basophil material associated with them which is argentophil.

#### DISCUSSION

The conclusions that follow from the results recorded above are these.

i. There exists in the neurones of vertebrates a three-dimensional reti-

culum, corresponding to the classical Golgi apparatus. The reticulum can be seen in living neurones by interference microscopy.

2. This reticulum consists of (a) irregular basophil masses, (b) thin basophil strands, and (c) non-basophil strands, which only occur in or near the axon. There are also (d) crescentic (or sometimes elongate) spaces in close association with the basophil material (that is to say, with (a) and (b)). These spaces also seem to occur in relationship with the non-basophil strands (fig. 12).

3. The irregular basophil masses are the Nissl bodies.

4. The irregular basophil masses and the two kinds of strands correspond with the endoplasmic reticulum of the electron-microscopists.

The contention, that the large Nissl bodies seen in the neurones of vertebrates after routine fixation and staining are the result of the action of fixative fluids upon a more dispersed substance in life, is based on the fact that they have not usually been seen in the living cell, even by phase-contrast microscopy (Malhotra, 1957a, 1958). Though Nissl bodies of the type seen after fixation cannot be visualized in the living cell by phase-contrast, there are reasons for believing that they exist as performed bodies in life, and they can be seen in freshly teased neurones by interference microscopy. This is in conformity with the conclusions of Palay and Palade (1955) and Palay (1956) who state that the electron microscope image of the Nissl bodies should be taken as an approximation of the situation in life. Weimann (1925), Bensley and Gersh (1933), Beams and King (1935), Beams and others (1952), Yamaoka (1953), Palay and Wissig (1953), Deitch and Murray (1956), and Deitch and Moses (1957) all believe in the pre-existence of the Nissl bodies in life. Pearce and Baker (1951) and Hartmann (1953) concluded that the Nissl bodies seen in their electron micrographs were gross fixation artifacts. This is not supported by my observations; nor is the conclusion of Koenig and Feldman (1954), based on observations by ultra-violet microscopy, that the ribonucleic protein of neurones is uniformly distributed in the cytoplasm.

It is commonly believed that the Nissl bodies are actually separate (Baily 1953; Maximov and Bloom, 1957). Nevertheless, the basophil substance in the neurones of vertebrates has been observed to form a continuous three-dimensional system in the cell body. It consists of large Nissl bodies connected with one another by strands, which seem to be made of similar material, but to have a higher refractive index.

The presence of basophil material forming a continuous net-like system in the neurones of vertebrates has been reported from time to time in the past, but it does not seem to have made any permanent impression. As far back as 1892 Vas described a network of chromatic substance in the cytoplasm of the sympathetic neurones of mammals. Later Sheinin (1932), who studied the effect of fixatives on the form and distribution of the basophil material, stated that this substance had a variable appearance. He recorded ‘irregular elongated granules with branching processes which anastomosed and gave the basophil substance the appearance of a coarse or fine network’. Similarly, Bensley and Gersh (1933) reported that the Nissl bodies were ‘often not

rely discontinuous with one another, the adjacent ones being connected by thin strands of similar material'. Dolley's (1913) illustration of the normal Purkinje cells of the rabbit (reproduced as fig. 4, B in the present paper) also indicates the presence of thin strands, connecting the Nissl bodies and forming a network. The recent studies of Palay and Palade (1955) and of Palay (1956) with the electron microscope also indicate this, as is clear from Palay's statement that the Nissl bodies are actually continuous with one another through strands of the same material and form a coarse meshwork.

The interference microscope has seldom, if ever, been used previously in the study of living neurones of vertebrates. This is probably the reason why so many authors have denied the existence of any network in the living cytoplasm of these cells (see p. 340 for references).

It is not possible to say whether Adamstone and Taylor (1953) saw any part of the Nissl-Golgi system in their studies of living neurones of the rat. It will be remembered that they used simple (direct) microscopy, not phase-contrast or interference. Their photomicrographs do not resemble what I have seen. I have never observed the movements within the cell described by these authors.

Interference microscopy has confirmed the existence of another component association with this reticulum formed by the Nissl bodies and the connecting strands, namely the canalicular spaces. These are the only structures of the Nissl-Golgi system that are distinctly visible by phase-contrast microscopy. In my earlier work I did not see them (1957a, 1958).

It seems probable that the canalicular Golgi apparatus reported by Gatenby and his associates (Gatenby, 1953, 1954; Gatenby and Moussa, 1949, 1950; Moussa, 1952, 1956; Moussa and Banhawy, 1954) corresponds to the spaces that I have seen by phase-contrast and interference microscopy.

Adamstone (1952) also described a similar canalicular Golgi reticulum in the neurones of the pig, but he interpreted the argentophil material as mitochondrial in nature. Cowdry (1913) also observed similar canal-like structures in neurones of the pigeon. These canals were blackened by the Mann-Kopsch method.

Penfield (1920) described the Golgi apparatus in fixed preparations as an attenuated reticulum with many varicosities or lacunae, which may frequently appear empty in silver preparations. Similar varicosities are also seen in Golgi's (1898a) figure depicting the ‘appareil réticulaire interne’ in the Purkinje cells of the cerebellum (fig. 4, A).

Apart from the lipid globules, the basophil strands are the only structures in the neurones of vertebrates that are readily darkened by both osmium and silver. It seems certain that the basophil strands have a greater affinity for osmium and especially silver than the Nissl bodies; and, therefore, the Golgi structures of the neurones of vertebrates are generally filamentous in silvered or slightly osmicated preparations. After longer osmication (about 5 to 7 days after fixation in Mann's fluid), massive bodies appear. These are mostly due to darkening of the Nissl bodies, which are known to consist of concentrations

of the endoplasmic reticulum with its associated basiphil granules (Palay and Palade, 1955; Palay, 1956). Some of them, however, represent the canalicular spaces, which become covered with a deposit of osmium. In silver preparations the Golgi apparatus is usually filamentous; and it seems that the silver salt is not reduced by the Nissl bodies. This is clearly demonstrated in the sympathetic neurones of the rabbit. In these cells the Golgi apparatus generally filamentous after silver methods, corresponding with the abundance of the basiphil strands in the ‘endoplasm’ (p. 356).

A comparison of Golgi’s (1898a) illustration, showing his apparatus in the Purkinje cells of the cerebellum of the barn-owl (fig. 4, A), with figs. 3 and 4, in the text, shows that his ‘appareil réticulaire interne’ is in fact a deposit of silver on the basiphil strands and the Nissl bodies. The latter seem to have been darkened by silver in this case. The dichromate-osmium / silver nitrate method used by Golgi for this investigation has not given me any constant results with the neurones of the pigeon. Later Golgi (1908) himself evidently realized that this technique was unreliable. In the dorsal root ganglion cell of the cat, also investigated by Golgi (1898b), the Golgi apparatus, which is in most places filamentous, seems to have been produced mainly by the silvering of the basiphil strands. Bulbous swellings of the type illustrated in the Purkinje cells of the barn-owl are not seen in the figures of the cat neurones.

It was Legendre (1910) who first pointed out the striking similarity, in the neurones of vertebrates, between the distribution of the Golgi apparatus, on one hand, and of the basiphil material, on the other. He considered that the Golgi apparatus was produced by the deposition of silver on the Nissl bodies. This important work of Legendre has been ignored and his suggestions have never been sufficiently considered. This is probably because the basiphil material and the Golgi apparatus have been dealt with by two different groups of research workers. The Golgi workers were not interested in the basiphil substance, while the neurologists, who mostly studied the basiphil material, were not much interested in the Golgi apparatus.

Legendre stimulated nerves by electric shock, and obtained similar alterations in the Golgi apparatus and Nissl bodies. In transplanted ganglia he noticed the disappearance of the Golgi apparatus and of the basiphil substance at the same rate. It is also known that in the chromatolysis resulting from direct injury, the Golgi apparatus and the basiphil granulations behave somewhat similarly: both move out to the periphery of the cell and sometimes disappear (Penfield, 1920).

Ramón y Cajal (1914) and Brambell (1923) agreed with Legendre that there was a similarity in the distribution of these two structures. Ramón y Cajal was reluctant to accept Legendre’s conclusions on the plea that the Nissl bodies were not known to occur in non-nervous cells.

Collin and Lucien (1909) seem to be the only authors who have seriously challenged Legendre’s findings, but their criticism is faulty. They give a figure of a cell of the cavy that has been silvered and stained with a basic dye.

The figure shows a silvered network in the endoplasm and dyed Nissl bodies at the periphery of the cell. They call the silvered network the Golgi apparatus, and distinguish between this and the Nissl bodies. However, in the text they say that the Nissl bodies in such cells extend throughout the cytoplasm. It would appear that in fact the basophil strands and Nissl bodies of the endoplasm have been blackened by silver, and this has prevented their being stained by the basic dye.

Beams (1931) has also given a figure of the d.r.g. cell of the rat prepared by the Mann-Kopsch method. This figure shows black filaments and greyish Nissl bodies (fig. 4, c). It seems that this cell has been lightly osmicated; and Beams has rightly interpreted the blackened threads as the Golgi apparatus. He did not realize, however, that they were basophil and connected with the Nissl bodies.

It seems certain that silver or osmium is deposited in Golgi preparations on the endoplasmic reticulum itself, not on the ‘small particles’ of Palade (1955a), which are usually associated with it. This follows from the fact that there are certain regions in the neurones, especially the axon, from which the basophil material is absent, but the Golgi apparatus extends into these processes in the form of narrow filaments (Gatenby and others, 1949, 1953). Recent studies with the electron microscope have shown that the endoplasmic reticulum is continued into the axon, but is here devoid of small particles of Palade (Thornburg, 1954; Palay, 1956). This is consistent with the fact that the threads that enter the axon are not basophil. It must, therefore, be inferred that the Golgi apparatus in its classical site, the neurones of vertebrates, is produced by the deposition of osmium or silver on the endoplasmic reticulum.

The ultra-centrifuge may throw the small particles of Palade to one pole without causing any significant movement of the Golgi apparatus. The reticulate structure of the latter makes it difficult to shift, as Brown (1936) realized. In a similar way, nerve stimulation may introduce different changes in the appearance of the Golgi apparatus and of the basophil substance.

It has not been possible to find in the living neurones, by interference microscopy, any cytoplasmic component that might correspond with the ‘granular reticulum’ of Palay and Palade (1955) and Palay (1956). The strands which are connected with the Nissl bodies cannot be the same as the agranular reticulum of these authors, because these are basophil. In the axon, however, these strands may be described as agranular, and this is in agreement with the absence of basophil substance in this region. Palade (1955b) and Palay (1958) state that the agranular reticulum is continuous with the endoplasmic reticulum, and the two thus constitute one system.

The absence of a reticular Golgi apparatus in the neurones of invertebrates may be correlated with the absence of distinct Nissl bodies and basophil strands (Young 1932; Beams and King, 1932). In conformity with this the evidence suggests that there is no aggregation of the endoplasmic reticulum into lumps in the neurones of invertebrates (De Robertis and Bennett, 1954). The endoplasmic reticulum is by no means always blackened in Golgi

preparations. Even in its massive form ('ergastoplasm') in the base of the exocrine cell of the pancreas, it does not reduce silver salts or osmium tetroxide, which in this case are reduced on another part of the cell. There is however, a case that is parallel to the neurones of vertebrates. Bennett (1956) and Porter (1956) have described in electron micrographs of striated muscle a system of vesicles and tubules linked together to form lace-like sleeves round the myofibrils. They homologize this system with the endoplasmic reticulum of other cells. They compare this with the reticulum recorded by earlier workers with the light microscope after silver and gold impregnation. In 1888 Ramón y Cajal clearly described it in fixed and in living tissue of the muscle of insects. Thin (1874) probably also saw it. Fusari (1895) certainly observed it in the striated muscle-fibres of insects and vertebrates, and it has often been called the Cajal-Fusari net. Later, when it was concluded that these nets were the representative of the Golgi apparatus in the muscle cell (see Bowen, 1926; Ramón y Cajal, 1933), Ramón y Cajal claimed that he had in fact discovered the Golgi apparatus long before Golgi.

Schlottke (1931), in his studies of the cytology of *Hydra*, maintained that the reduction products of osmium tetroxide were deposited indiscriminately on essentially different objects in different kinds of cells. MacLennan (1941) found that in Protozoa the term Golgi body included a heterogeneous group of structures, and that the retention of the term Golgi body was merely convenience to bridge the change from reliance on the non-specific osmium techniques to reliance upon cytochemical and physiological criteria. Hibbar (1945) also stated that more than one category of inclusions were blackened by Golgi techniques and described under the name of Golgi apparatus. Since 1950 Baker (1950, 1953b, 1957a) has been urging that the use of the term Golgi apparatus should be given up, because objects which are essentially different in different kinds of cells are being grouped under this name. Natt (1957) and I (1957a, 1958) have also recently adopted this view.

The reticulum described in the present paper is obviously not homologous with the bodies commonly called dictyosomes or Golgi apparatus in the neurones of many invertebrates (Moussa, 1950; Lacy and Rogers, 1956). They do not resemble the Nissl-Golgi system either at the light microscopic level or in their ultrastructure. The dictyosomes are lipid droplets (Shafiq 1953, 1954; Chou, 1957; Chou and Meek, 1958; Malhotra, 1956, 1957b, c) which correspond in a general way with the lipid droplets of neurones of vertebrates (Thomas, 1951; Shafiq and Casselman, 1954; Casselman and Baker, 1955).

The term 'Nissl body' is usually used to mean the massive, irregular bodies that Nissl (1894b) called *Körperchen*. Nissl, however, clearly saw the basiphilic strands or *Fäden* and illustrated them repeatedly (e.g. 1894a). He knew that the strands often anastomosed to form a reticulum (*Netz*, Nissl, 1894a, p. 67, and fig. 2; see fig. 13 of the present paper). It has not previously been realized that this reticulum is visible in living neurones by interference microscopy.

It is probable that in Nissl preparations the basic dyes colour the small

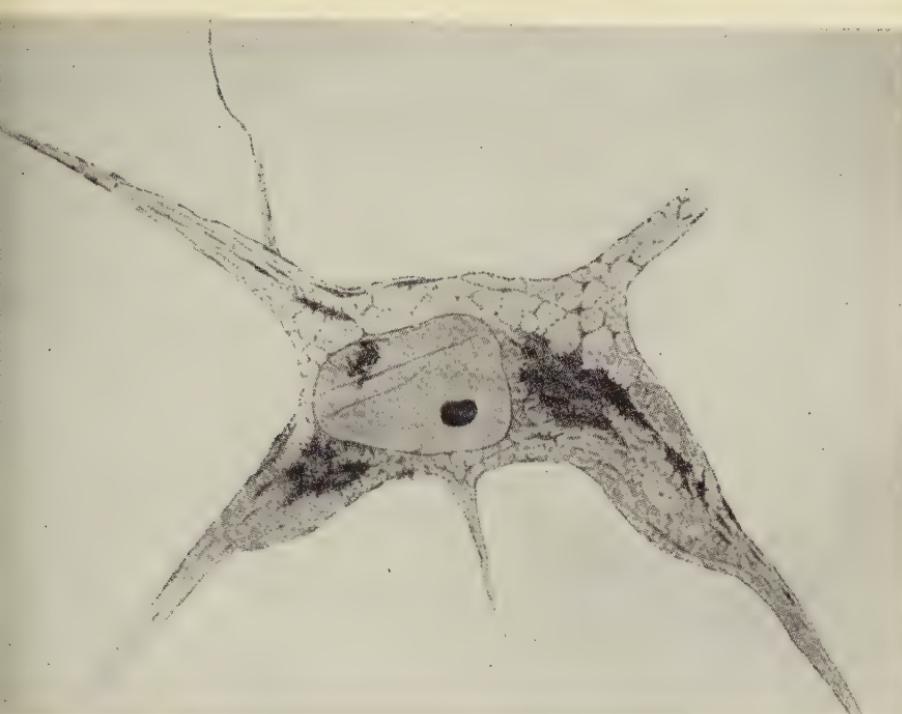


Fig. 13. Cell from a dorsally situated nucleus in the proximal part of the medulla of rabbit to show the basophil reticulum. Reproduced from Nissl (1894a).

articles of Palade, which are scattered over the surfaces of the endoplasmic reticulum. It is unlikely that these particles reduce silver nitrate or osmium tetroxide. It is more likely that it is the endoplasmic reticulum itself that blackens in the Golgi techniques. Thus *the same strands and bodies* are shown by the Nissl and Golgi techniques (except in the axon), but different components are responsible for the two reactions. There is no purpose in giving two different names to the strands and bodies, according to whether they have been dyed by a basic dye or blackened by the deposition of a metal.

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# Histological and Histochemical Study of the Brown and Yellow Adipose Tissue of the Bat, *Hipposideros speoris*

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With two plates (figs. 1 and 2)

## SUMMARY

A study of the histology and histochemical reactions for lipase, alkaline phosphatase, adenosine triphosphatase, succinic dehydrogenase, lactic dehydrogenase, phospholipids, cholesterol, sulphhydryl groups, and water-insoluble aldehydes and ketones in the brown and yellow adipose tissue of the bat (*Hipposideros speoris*) revealed that the two types of adipose tissue differ in histological structure as well as physiological activity. The histological structure of the two types of adipose tissue was found to be different, resembling that of the two corresponding types of the rat. The brown adipose tissue showed a higher concentration of succinic dehydrogenase, lactic dehydrogenase, phospholipids, cholesterol, and sulphhydryl groups. No detectable difference between brown and yellow adipose tissue was, however, found with respect to lipase, alkaline phosphatase, acid phosphatase, adenosine triphosphatase, and water-soluble aldehydes and ketones.

## INTRODUCTION

THE occurrence of two types of adipose tissue, brown and yellow, in certain mammals is well known and they have been the subject of detailed studies in recent years (Mirski, 1942; Fawcett, 1952; Menschik, 1953; Sidman, 1956; Rothbard, 1958; Remillard, 1958). They are different in their histological structure as well as physiological activity. Sidman (1956) has reported that brown adipose tissue does not differ fundamentally from the yellow with regard to development and also contended that intergrades between them occur. In a few cases like that of rabbit, cat, and man, much of the brown adipose tissue which is distinctly multilocular in the embryonic stages tends to become unilocular and to resemble the yellow type. But in the majority of other mammals (rodents and many hibernators) possessing brown adipose tissue in earlier stages retain the same condition in adult life also (Selye and Timiras, 1949; Sidman, 1956). The brown and yellow adipose tissues are named multilocular and unilocular respectively with reference to the number and size of the lipid droplets present in the individual cells. The physiological differences appear, however, to be mainly quantitative rather than qualitative. Fawcett (1952), using histochemical methods, suggested that there are quantitative differences in enzymic activities between brown and yellow adipose tissue of rats and claimed that brown adipose tissue is more active. Qualitative differences were not detected. On the other hand, Mirski (1942) had reported one instance of qualitative difference between brown and yellow adipose tissue of the rat, namely the absence of the enzyme phosphoglucomutase in the latter. In bats also these two types of adipose tissue are present. A histochemical

and microchemical study of the lipids of the interscapular brown adipose tissue of the vespertilionid bat, *Myotis lucifugus lucifugus*, was made by Remillard (1958). So far no data are available on the relative activities of these two types of adipose tissue of the bat. In the present paper the results are reported of a comparative study of the histological organization and histochemical reactions of lipase, alkaline phosphatase, acid phosphatase, adenosine triphosphatase (ATPase), succinic dehydrogenase, lactic dehydrogenase, phospholipids, cholesterol, sulphhydryl groups, and water-insoluble aldehydes and ketones of the brown and yellow adipose tissue of the bat (*Hipposideros speoris*).

#### MATERIAL AND METHODS

The materials used were subcutaneous lobes of interscapular brown and lateral abdominal yellow adipose tissue of the bat (*H. speoris*). The animals were decapitated and the tissues taken after most of the blood had been drained off. Fresh frozen sections were used for the study of enzymes and also for certain other histochemical observations. For studying the normal histology, tissues were fixed in Zenker's fluid for 1 h, washed in running water for the same time, and dehydrated in the usual manner. Paraffin sections were cut at 10  $\mu$  and stained with haematoxylin and eosin.

Lipase activity was studied by employing the 'tween' method of Gomori (Pearse, 1954). 'Tween 80' was used as substrate. Sections were mounted on clean, dry slides and allowed to remain at room temperature for about 1 min to ensure adherence. This was employed in all the other enzyme studies also unless otherwise stated. Before fixation sections were deprived of their fats by soaking in ethyl ether for 10 min. This modification was necessary because sections not treated in this way did not yield good results (George and Eapen, 1958). The same procedure was adopted in studies of all other enzymes except succinic and lactic dehydrogenases. Sections were fixed in cold (4° C) 6% neutral formalin for 16 h. After fixation the sections were washed in running water for 1 h and then rinsed in distilled water and incubated at 37° C for 8 h. The incubation mixture contained 5 ml 0.1 M bicarbonate buffer at pH 8.4, 2 ml 10% calcium chloride, 2 ml 5% 'tween 80', 4 ml distilled water, and a crystal of thymol as preservative (George and Scaria, 1958). The rest of the procedure was the same as described by Pearse (1954). The sections were not counterstained. Sections boiled in distilled water before incubation were used as control.

Alkaline phosphatase was studied according to the revised method of Gomori, sodium glycerophosphate being used as substrate. Sections previously extracted with ethyl ether were fixed in cold 10% neutral formalin for 1 h, washed in running water for 30 min, rinsed with distilled water, and incubated in the substrate medium (Pearse, 1954) at 37° C for 24 h. The rest of the procedure was the same as described by Pearse (1954). Sections which had been kept in boiling water for 10 min, before incubation were used as control. Another control employed was the incubation of sections in the incubation medium devoid of the substrate (sodium glycerophosphate).

The revised method of Gomori was successfully employed on fresh frozen sections of adipose tissue to demonstrate acid phosphatase activity (Glick, 1949). After extraction with ether, sections were fixed in cold ( $4^{\circ}\text{C}$ ) 10% formalin for 1 h, washed in running water for 1 h, rinsed in distilled water, and incubated for 6 h, at  $37^{\circ}\text{C}$ . The incubation mixture was prepared as described by Glick (1949). The stock solution was filtered and diluted with ice the amount of distilled water before use. Incubated sections were rinsed well in distilled water and kept for a few min in 2% acetic acid. After rinsing again in distilled water the sections were placed in diluted ammonium sulphide and then washed in distilled water and mounted in glycerine jelly. Boiled sections were used as control.

ATPase was detected by the procedure of Pearse and Reis (Pearse, 1954). Sections were fixed in cold 10% neutral formalin for 1 h, washed in running water for the same time, rinsed in distilled water and incubated for 4 h at  $37^{\circ}\text{C}$ . The incubation medium used was the same as described by Pearse (1954). After incubation, sections were washed with 2% calcium nitrate, rinsed in distilled water, and treated with 2% cobalt nitrate for 5 min. Sections were thoroughly washed in running water and then in distilled water, and treated with diluted ammonium sulphide. After washing, the sections were mounted in glycerine jelly. A few sections were incubated as for alkaline phosphatase at pH 9.2 and for alkaline phosphatase at pH 7.5, and in a similar medium as for ATPase but containing distilled water in place of the substrate (Pearse, 1954). In addition sections boiled in distilled water before incubation were also used as control.

Succinic dehydrogenase activity was studied by the method of Straus and others (Pearse, 1954), 2:3:5 triphenyl tetrazolium chloride (TTC) being used as the hydrogen acceptor. The same method was extended to the study of lactic dehydrogenase also. Fresh frozen sections were cut into cold 1 M phosphate buffer at pH 7.2. After being kept in the buffer for 15 min to ensure that all the endogenous activity was lost, they were transferred to the respective incubation mixtures in cuvettes and incubated for 15 min at  $37^{\circ}\text{C}$ . The incubation mixture in each case contained 1.5 ml of phosphate buffer at pH 7.2, 1 ml of 0.1 M solution of the substrate, 7.5 mg of TTC, and 0.625 mg of DPN in a total volume of 2.5 ml. DPN was omitted from the incubation mixture of succinic dehydrogenase because this enzyme does not require the coenzyme (Baldwin, 1957). A few sections were incubated in an incubation medium without the substrate. These served as control.

Phospholipids were demonstrated by the acid haematein method of Baker. Baker's pyridine extraction test was applied as control (Pearse, 1954). For the study of cholesterol, the Schultz method (Pearse, 1954) was employed. Aldehydyl groups were detected by Bourne's nitroprusside test (Glick, 1949), and water-insoluble aldehydes and ketones by the Albert and Leblond reaction (Glick, 1949).

## OBSERVATIONS

Fig. 1 shows sections of brown and yellow adipose tissue stained with haematoxylin and eosin. In the brown adipose tissue (A) the cells are smaller, the cytoplasm more abundant, and nuclei more centrally situated. The fat droplets in the cytoplasm do not become confluent and hence the cytoplasm has a vacuolated appearance. The cells of the yellow adipose tissue are many times bigger and their nuclei lie at the periphery.

*Lipase.* Sections treated for lipase activity showed more or less equally abundant precipitate in both the tissues (fig. 1, C, D). The brown adipose tissue with its larger amount of cytoplasm did not show any appreciable difference in this respect. Lipase activity was represented by a brownish precipitate.

*Alkaline phosphatase.* The intensity of the brown colour developed was almost the same or perhaps slightly more in the case of the brown adipose tissue.

*Acid phosphatase.* The activity of this enzyme in the two types of adipose tissue appeared to be almost the same. Fig. 2, A, B shows brown and yellow adipose tissue treated for acid phosphatase activity. The nucleus as well as the cytoplasm showed the presence of this enzyme, but the reaction in the former was stronger.

*ATPase.* Fig. 2, C, D shows sections of the brown and yellow adipose tissue treated for ATPase activity. The intensity of the brown colour developed after the final treatment was found to be almost the same in both the types of adipose tissue. Both the nucleus and the cytoplasm gave a positive reaction, that in the former being stronger.

*Dehydrogenases.* Succinic dehydrogenase and lactic dehydrogenase activities in these two types of adipose tissue showed a sharp difference. The brown adipose tissue showed a much higher activity. Sections of brown adipose tissue after a few minutes of incubation developed a deep red colour in contrast to a light pink in the case of the yellow adipose tissue.

*Phospholipids.* The brown adipose tissue revealed the presence of a high concentration of phospholipids (fig. 2, E, F).

*Cholesterol.* Both brown and yellow adipose tissue after treatment gave

FIG. 1 (plate). Photomicrographs of sections of the brown and yellow adipose tissue of the bat, *H. speoris*.

A, the brown adipose tissue. The cytoplasm has a vacuolated appearance. The nuclei are eccentric. (Haematoxylin and eosin.)

B, the yellow adipose tissue. The cytoplasm is confined to the periphery of the cell, the inside being filled by a single, large fat drop. The nucleus occupies a peripheral position. (Haematoxylin and eosin.)

C and D, sections of the brown and yellow adipose tissue respectively, treated for lipase activity. The abundance of the brownish precipitate indicates high activity of the enzyme. The individual cells are obscured by the precipitate. (Gomori's 'tween' method, with 'tween 80' as substrate, incubated for 8 h.)

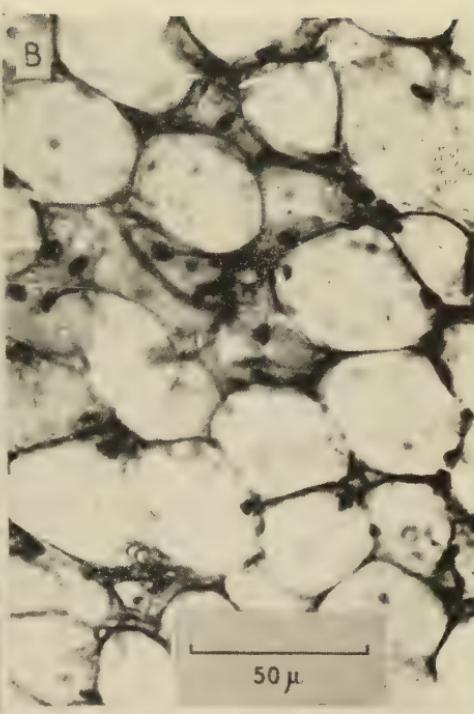
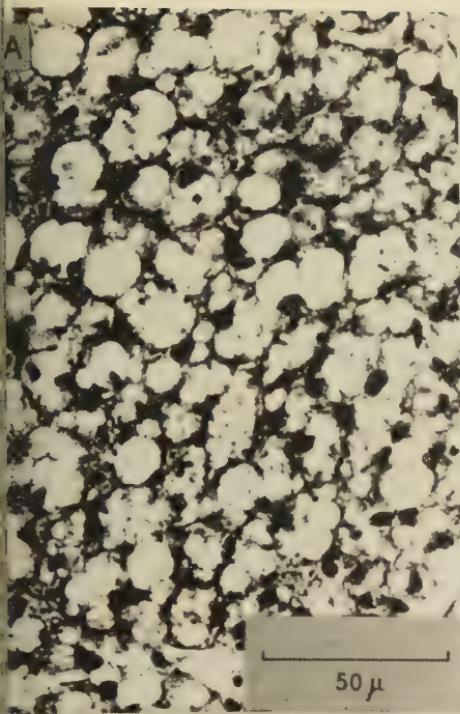


FIG. 1

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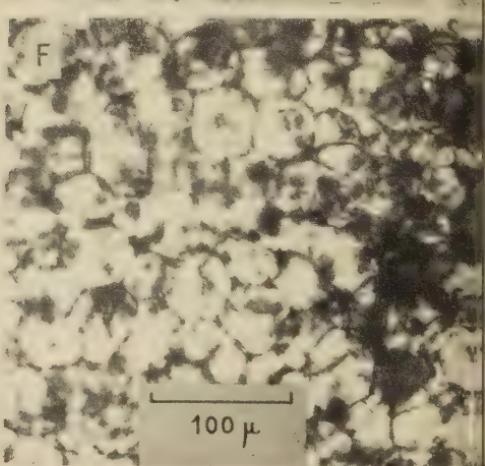
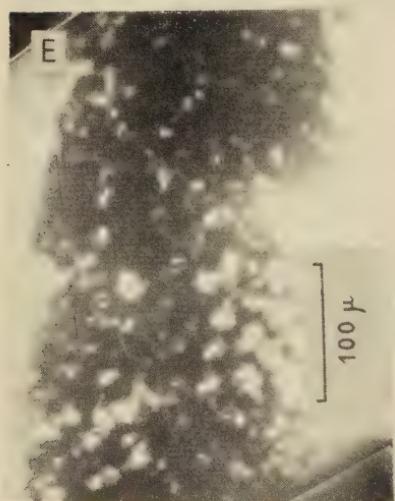
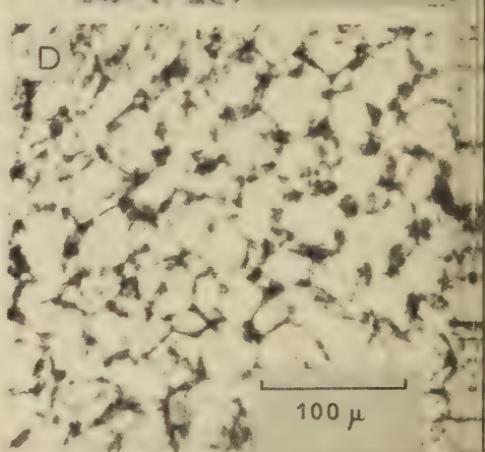
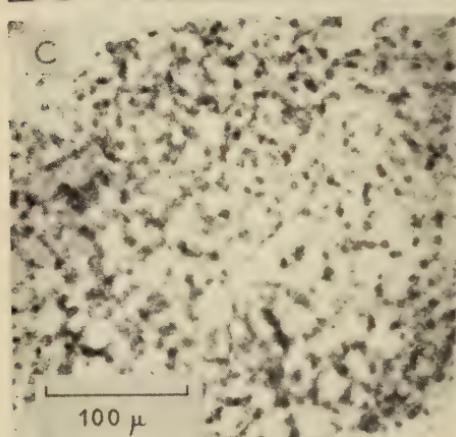
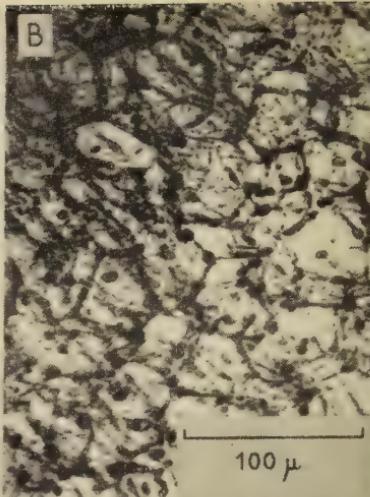
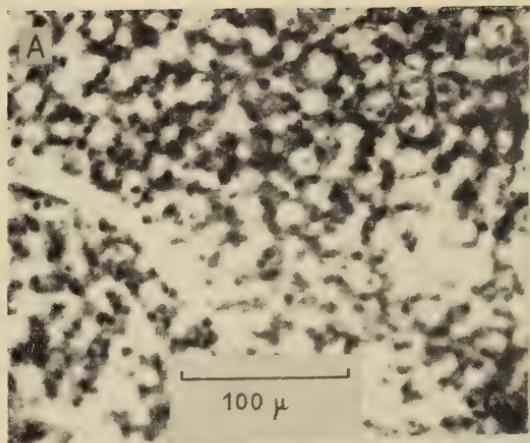


FIG. 2

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ry faint green coloration, indicating a positive reaction. The intensity of the colour developed was slightly greater in the brown adipose tissue.

*Sulphydryl groups.* The brown adipose tissue showed the presence of a higher concentration of sulphydryl groups than the yellow.

*Water-insoluble aldehydes and ketones.* Brown and yellow adipose tissue gave almost the same reaction when tested for water-insoluble aldehydes and ketones.

### DISCUSSION

Histological differences between the two types of adipose tissue are quite distinct. The amount of cytoplasm present is very much greater in the brown adipose tissue than in its counterpart. In structure both the brown and yellow adipose tissue of this bat more or less resemble the corresponding tissues of the rat, as described by Fawcett (1952).

The development of an abundant deposit of precipitate in sections treated for lipase activity indicates that both these types have appreciable concentrations of lipase in them. Fawcett (1952) has shown by histochemical methods that the brown adipose tissue of the rat has a higher concentration of lipase than the yellow adipose tissue. However, he expressed doubt as to the presence of 'true lipase' in the adipose tissue of the rat, since he used 'tween 40' and 'tween 60' as substrates. It should be mentioned here that 'tween 80' was found to be attacked specifically by 'true lipase' and not esterase (Gomori, 1953). Since in our present study we have used 'tween 80' as the substrate, it is concluded that the enzyme under consideration is a 'true lipase'. By histochemical as well as quantitative methods we have shown (1958) the presence of 'true lipase', in the adipose tissue of the pigeon.

The distribution of alkaline phosphatase, acid phosphatase, and ATPase is found to be almost the same in the two types of adipose tissue. Fawcett (1952) reported higher concentration of alkaline phosphatase in the brown

FIG. 2 (plate). Photomicrographs of sections of the brown and yellow adipose tissue of the bat, *H. speoris*.

a, the brown adipose tissue treated for acid phosphatase activity. The nuclei showed a higher activity than the cytoplasm. (Gomori's method, sodium glycerophosphate substrate, incubated for 6 h.)

b, the yellow adipose tissue treated for acid phosphatase activity. The cytoplasm appeared to be more active than the cytoplasm of the brown adipose tissue cells. However, the nucleus was found to contain less of the enzyme when compared to the nucleus of the other type of tissue. (Gomori's method, sodium glycerophosphate substrate, incubated for 6 h.)

c, the brown adipose tissue, demonstrating ATPase activity. The nucleus showed greater activity than the cytoplasm. This was true for the yellow adipose tissue cells also. (Method of Pearse and Reis, ATP substrate, incubated for 4 h.)

d, the yellow adipose tissue, demonstrating ATPase activity. The cytoplasm showed greater activity than the cytoplasm of the brown adipose tissue cells. (Method of Pearse and Reis, ATP substrate, incubated for 4 h.)

e, the brown adipose tissue treated to reveal phospholipids. The cytoplasm gave a dark blue colour, showing a high phospholipid content. (Baker's acid haematein method.)

f, the yellow adipose tissue treated for phospholipids. The thin film of cytoplasm at the periphery of the cell gave a positive result. The remaining part of the cell remained unaffected. (Baker's acid haematein method.)

adipose tissue than in the yellow in the rat. Both nucleus and cytoplasm gave positive result. In a number of sections examined, the cytoplasm of the yellow adipose tissue appeared to be more active than that of the brown adipose tissue, and conversely in the case of the nuclei. This cannot be explained within the limits of our present state of knowledge.

The presence of greater concentrations of succinic and lactic dehydrogenase in the brown adipose tissue is indicative of its higher oxidative activity. It is likely that the other oxidative enzymes are also more abundant in the brown adipose tissue. This is supported by the findings of various workers which have been reviewed by Remillard (1958). Fawcett (1952) reported high succinic dehydrogenase activity in the brown adipose tissue of the rat while Menschik (1953) found no difference between brown and yellow adipose tissue of the guinea-pig. However, the latter found a higher concentration of amine oxidase,  $\alpha$ -naphthol oxidase, and cytochrome oxidase in the brown adipose tissue.

Fawcett (1952) found higher concentration of phospholipids in the brown adipose tissue of the rat and Menschik (1953) in the guinea-pig. We too have obtained a similar result in the case of the bat. Fawcett (1952), however, failed to get a positive result for cholesterol in both types of adipose tissue in the rat, whereas Menschik (1953) reported the presence of more cholesterol and its esters in the brown adipose tissue of the guinea-pig. In the bat we get a faint reaction in both types of adipose tissue.

The brown and yellow adipose tissue showed a striking contrast when tested for sulphhydryl groups, the former showing an abundance. In the guinea-pig Menschik (1953) found no difference in reaction in the two types of adipose tissue.

The question arises whether these two varieties of adipose tissue are functionally the same or not. A definite answer to this is not yet available. Fawcett (1952) suggested in his paper that fat in the brown and yellow adipose tissue is different in chemical nature. The former is said to contain more saturated fat and phospholipids while the latter contains more neutral fat. There appear to be seasonal variations also with regard to the amount and nature of the fat present. Remillard (1958) has stated that the fat of the yellow adipose tissue is mobilized in a greater amount faster than in the brown adipose tissue. Rothbard (1958) attributes a dual function to the brown adipose tissue in the metabolism of the mouse. He considers that it is needed in milk production and as energy source in the process of parturition. The function of this tissue in the male remains unexplained.

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# Neurosecretion in the Brain of the Larva of the Sheep Blowfly, *Lucilia caesar*

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## SUMMARY

Six groups of neurosecretory cells were identified in the brain of the mature larva *Lucilia caesar*. Five of these groups belonged to the category of medial neurosecretory cells and one to that of lateral neurosecretory cells. The groups differ in position, cell size, staining characteristics, and sequence of activity. It is apparent that only some of the groups are concerned with the process of thoracic gland activation, though all are active during metamorphosis. At least one group, not concerned in thoracic gland activation in the non-diapause larva, is continually active during diapause.

## INTRODUCTION

It is accepted that in the brain of the pterygote insect the neurosecretory cells lying in the protocerebrum form two medial and two lateral groups. Hanström (1940) observed that the axons of the two medial groups form two nerves which in their intracerebral course cross over each other in the pars intercerebralis and, on leaving the rear of the brain, form the *nervi corporis cardiaci interni* (NCC 1), while the axons of the lateral cells take a direct course to the rear of the brain and externally form the *nervi corporis cardiaci externi* (NCC 2).

In the larvae of Diptera-Cyclorrhapha the NCC 1 and NCC 2 on each side unite to form a single pair of nerves to the corpus cardiacum. M. Thomsen (1951) and Possompès (1953) described neurosecretory cells in the pars intercerebralis of the larva of *Calliphora erythrocephala* Mg. The former identified these cells as belonging to the medial group, but lateral neurosecretory cells have not been identified hitherto in cyclorrhaphan larvae.

The phenomenon of transport of neurosecretory product from the brain to the corpus cardiacum was first noted by Hanström (1940) and has since been confirmed by the use of various selective stains in a large number of species. Experimental confirmation of axonal transport of the product has been provided by Scharrer (1952) and E. Thomsen (1954). Scharrer (1951) showed that this material is stored in the corpus cardiacum of *Leucophaea* near the origin of the aorta and postulated that it was released thence into the blood. According to M. Thomsen (1954) the axons of the NCC of Hymenoptera emerge in the corpus cardiacum and branch between the cells, secretion granules being stored in the swellings of the axons. It has also been noted in a number of species that some of the axons in the NCC do not terminate in the corpora cardiaca but pass through and form the nerves to the corpora allata. Transport of neurosecretory material to and accumulation of it in the corpora

allata have been described by Arvy, Bounhiol, and Gabe (1953), Arvy and Gabe (1953a), and M. Thomsen (1954).

The secretory cycle in these neurones in relation to post-embryonic development has been studied in *Ephestia* by Rehm (1951), in Ephemeroptera, Odonata, Plecoptera, and in *Tenebrio* by Arvy and Gabe (1953 a, b, c, d), *Forficula* by Lhoste (1953), and in *Bombyx* by Arvy, Bounhiol, and Gabe (1953). Gabe (1954), summarizing the available information, stated that in Holometabola each larval stage is marked by a phase of secretion in the protocerebral intercerebralis.

The objects of this study were (a) to locate and identify the neurosecretory cells in the brain of the mature larva of *Lucilia caesar* L.; (b) to determine, by means of a variety of staining procedures and histochemical tests, the chemical nature of the stainable product in these cells; (c) to attempt to determine the function of the neurosecretory cell groups by relating histological evidence of activity in these at different stages in development to histological, developmental, and experimental evidence of activity in extracerebral endocrine organs.

#### METHODS

Serial sections were prepared of the brains of third instar larvae of *L. caesar* taken at the following stages in development.

*Diapause stage.* A facultative diapause occurs in this species after the cessation of feeding and before puparium formation.

*Stage 1.* Large third-instar larvae, still feeding but by their size judged to be due to cease feeding within a few hours.

*Stage 2.* Larvae taken 6 to 8 h after the cessation of feeding, at which time cannot be determined whether a larva will enter diapause or develop directly.

*Stage 3.* Mature larvae developing without diapause, selected early in the post-feeding pre-pupation phase when the imaginal discs are very slightly larger than those of diapause larvae in which *Anlagen*-development is arrested. These larvae had spent the 72 h since removal of food in optimal conditions. That a larva was not in diapause could only be proved by the evidence, i.e. sections, of mitoses in *Anlagen*.

*Stage 4.* Mature larvae developing without diapause, in which development of *Anlagen* is marked.

*Stage 5.* This is the 'white pupa' stage at which the cuticle of the larva is contracted to form the puparium but has not yet darkened.

Bouin's solution was used to fix all the material sectioned except that required for the Sudan black B test, for which a formaldehyde-calcium fixative is recommended. Steedman's (1947) ester wax was the embedding medium used and sections were cut at 6  $\mu$ .

The following staining procedures, of which the first two are somewhat selective stains for the neurosecretory product, were employed.

(1) The chrome-haematoxylin stain of Gomori (1941), after oxidation of sections with acidified 0.3% potassium permanganate solution, with phlor-

unterstaining. With this stain granules in neurosecretory cells appear dark blue-black.

(2) The paraldehyde-fuchsin stain of Gomori (1950) as adapted by Gabe (1953), with preliminary oxidation with acidified potassium permanganate, unstained with Groat's haematoxylin and picro-indigocarmine. The granular product of neurosecretory cells, basement membranes, material in the pore canals of the fore gut intima and a part of the perineurium all stain a brilliant purple colour. Gomori (1952) states that acidified potassium permanganate solution will attack glycol linkages, the oxidant converting 1:2 glycol groups to dialdehydes in the first stage of oxidation. The selective coloration of neurosecretory product by chrome-haematoxylin and by paraldehyde fuchsin, after acidified permanganate oxidation, was considered by Gabe (1955) to be due to the affinity of these stains for aldehyde and sulphydryl groups liberated by the oxidation. While it was recognized that this oxidant is able to oxidize further the resulting aldehydes, it seemed a reasonable working hypothesis that these 1:2 glycol groups were associated with dehydroglucose residues of polysaccharide molecules and that a component at least of the neurosecretory product could fit into the general classification of 'carbohydrates' as used by Pearse (1953). Several 'carbohydrate' staining procedures, detailed below, have therefore been used in an attempt to identify this component, such an attempt being justified by Gabe's (1954) general statement that the product of insect neurosecretory cells has a PAS-positive component other than glycogen.

(3) Steedman's (1950) alcian blue was first developed as a stain for mucin and is of use (Pearse, 1953) in differentiating acid mucopolysaccharides from other carbohydrates. It was used in combination with either neutral red or hemalum-eosin counterstaining. Positively stained material has a brilliant blue-green colour.

(4) The standard toluidine blue metachromatic method for identification of carbohydrates as described by Pearse (1953) was used. Metachromatic substances are either red-pink ( $\gamma$  metachromasia) or purple ( $\beta$  metachromasia).

(5) The periodic acid / Schiff (PAS) test for polysaccharides. Exhaustive discussions of the PAS reaction are to be found in Gomori (1952) and Pearse (1953), the latter listing polysaccharides, mucopolysaccharides, mucoproteins, glycolipids, unsaturated lipids, and phospholipids as giving a positive reaction. McManus (1956) cautions that a variety of substances apart from those containing 1:2 glycol linkages have been found to give a positive reaction but he quotes data from organic chemistry to reinforce the belief that, provided that oxidation is performed with 0.5% periodic acid solution for not longer than 5 min, positive substances are presumably carbohydrates. Several workers have recommended a reducing rinse immediately after periodic acid oxidation, but as McManus logically points out this can only result in the reversal of the oxidation just performed. Glycogens give a positive reaction, but can be readily eliminated by pretreatment of sections with diastase solution or ptyalin. Positive material stains a rich red or purple-red colour.

(6) Sudan black B staining for lipids in wax sections (Pearse, 1953) after McManus (1946). Since the preparation of serial frozen sections from minute tissue blocks like the larval brain presents extreme technical difficulties, this procedure was adopted. The tissue was fixed for 3 weeks in a formaldehyde-calcium solution and then postchromed for 24 h; thereafter the routine embedding procedure for ester wax was used. Lipids are coloured blue or blue-black.

(7) Lillie (1950) found that the contents of cells of the mammalian adrenal medulla were phenolic and PAS-positive. The acid diazonium reaction for aromatic amines and phenols (Pearse, 1953) was therefore used to test whether certain neurosecretory cells containing PAS-positive material might contain a phenolic substance.

## RESULTS

The larval brain is invested in a sheath formed by a layer of cells called the perineurium (Scharrer, 1939), which is in turn covered by a thin neurilemma. The medullary zone of the brain is composed principally of axons, while in the cortical zone are found neurones, neuroglia, and the *Anlage* cells, from which certain structures of the adult brain ultimately develop. Some of the neurones in the cortex are identified as neurosecretory. At some stage in the development of the larva they can be distinguished during life from common neurones by a characteristic bluish appearance. They prove, in appropriately stained sections, to contain a particulate product. The refractive properties of the product are responsible for the colour observed in the living cell (E. Thomsen, 1954).

No fewer than 6 groups of these cells can be identified in each hemisphere of the brain of *L. caesar* larvae. The cells are all unipolar pyriform neurones located as follows (fig. 1).

*Group 1* consists of two large cells lying in an antero-dorsal position in the pars intercerebralis close to the junction of the hemispheres. They attain a maximum size of about  $40 \times 18 \mu$  and each cell contains numerous ovoid vacuoles ranging in size up to  $4 \mu$  in length.

*Group 2* is represented by a single cell lying anterior to Group 1 and at a lower level. It measures  $20 \times 15 \mu$ . Vacuoles are normally seen in cells of this group.

*Group 3*. Four cells can normally be counted in this group. They lie laterally to Group 1 in the dorsum of the hemisphere and measure  $20 \times 15 \mu$ . Two or three large vacuoles are usually seen in the fixed cell.

*Group 4*. As indicated in fig. 1, the cells of Group 4 have the same location as, and in fact intermingle with, those of Group 3, but the two groups have distinct characteristics. There are usually 7 Group 4 cells, each distinctly pyriform with the nucleus displaced toward the axon end of the cell. The maximum size is  $25 \times 17.5 \mu$ .

*Group 5* is formed of small almost spherical neurones, about  $12.5 \mu$  in diameter, lying laterally to and behind Groups 3 and 4. Several morphologically

ically similar neurones occur in the dorsolateral locus but the actual number differentially stained varies from 4 to 8.

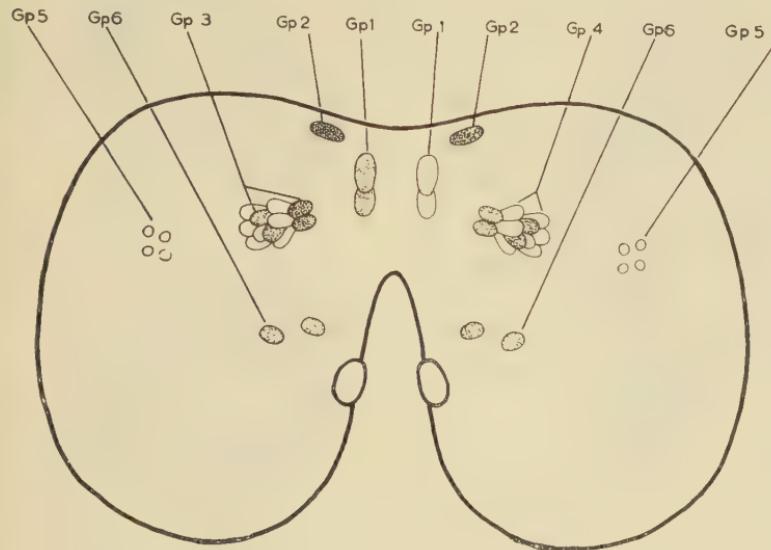


Fig. 1. Diagram showing the distribution of neurosecretory cell groups in the dorsum of the brain of the larva of *L. caesar*.

Group 6 comprises two cells of irregular outline, about  $20 \times 17.5 \mu$  in size. These lie against the medulla at the rear of the brain, close to the entry of the main cerebral tracheae.

#### Staining characteristics of the neurosecretory cell groups

*Paraldehyde-fuchsin.* In brains of larvae in diapause the cells of Group 1 (fig. 2, A) appear at low magnification to have a uniform deep purple colour broken only by the nucleus and by the several large vacuoles. At higher magnifications this colour is resolved into minute purple particles, the smallest of which are at the resolution limit of the microscope. The small particles appear to associate to form aggregates or granules of varying magnitude but none of these exceed  $1 \mu$  and most are half that size. The general impression is of a very high concentration of small separate granules packing a distended cell. In non-diapause brains these cells have at Stage 1 a grey cytoplasm, like that of common neurones. Then the first purple particles appear, just detectable around the periphery of the vacuoles. At the second stage (fig. 3, A) the amount of product has increased and the deep purple granules around the vacuoles are larger. Although granules are found scattered in all parts of the eukaryon, they are densest in the vicinity of the vacuoles. During Stage 3 the granules remain limited in size but increase in abundance (fig. 3, B, C). There continues to be marked association with vacuoles, which appear to be centres of production from which the material spreads out into the surrounding cytoplasm. Late in this stage particles of product can be traced into and

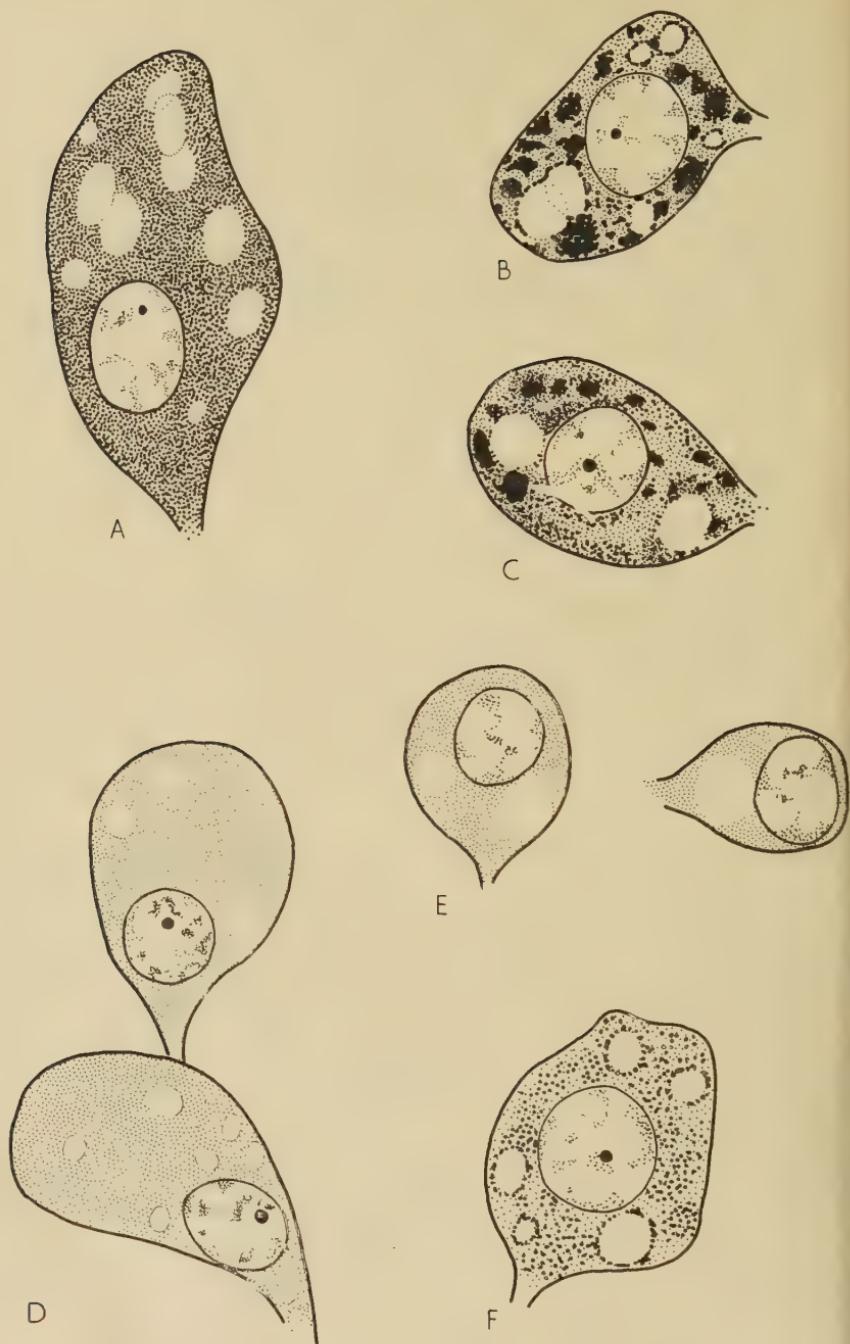


FIG. 2. Neurosecretory cells in the brain of an *L. caesar* larva in diapause, stained with paradehyd-fuchsin. A, Group 1. B, Group 2. C, Group 3. D, Group 4. E, Group 5. F, Group 6.

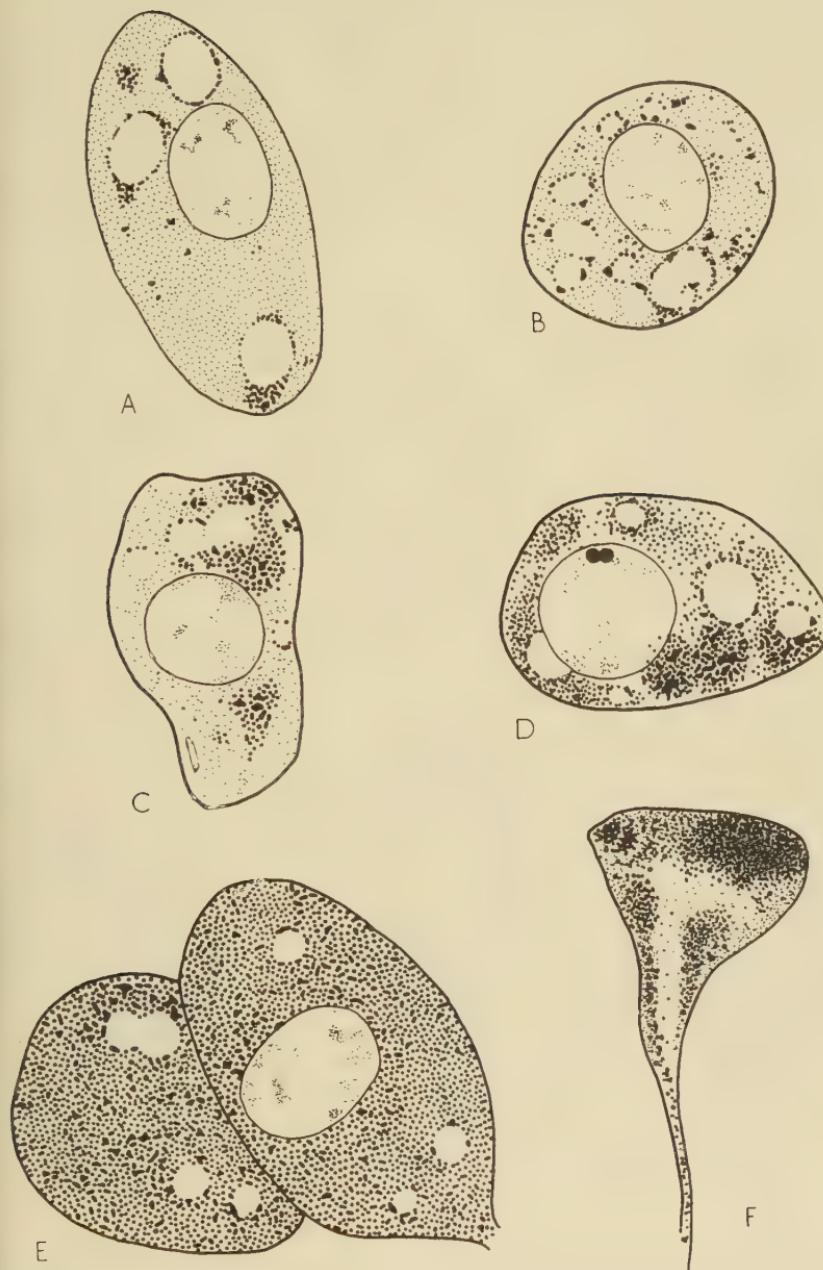


FIG. 3. Cells of Group 1; paraldehyde-fuchsin staining. A, Stage 2. B, early Stage 3. C, late Stage 3. D, early Stage 4. E, late Stage 4. F, Stage 5.

along the axons of cells of the group. Early in Stage 4 the product is dense in sub-spherical zones around the vacuoles (fig. 3, D). Later the entire cell, now noticeably distended, is, except for vacuoles and nucleus, entirely filled with minute separate granules (fig. 3, E). There is a pronounced similarity between late Stage 4 cells and those in diapause brains. In the final Stage the cell collapses. The nucleus is pycnotic and the cell outline irregular (fig. 3, F). It is clear that in Stage 4 the rate of production is greater than the rate of secretion which started in Stage 3. In the last stage, production has ceased and the product is being transmitted along the axon in much greater amount than at previous stages.

The product of Group 2 (fig. 2, B) occurs, in diapause brains, in larger granules than in Group 1, and these granules associate in large, irregular masses separated by relatively clear zones of cytoplasm. In the younger feeding larva there is no positive material in cells of this group but this is the first group in which secretory material is formed after the cessation of feeding (fig. 4, A). At Stage 2 the granules form distinct aggregates but there is no marked association with vacuoles (fig. 4, B, C). During Stage 3 aggregates continue to increase in size (fig. 4, D). There are also numerous unassociated granules, scattered in the cytoplasm, larger than those in Group 1. Particles of product occur in the axons at this stage but the rate of discharge is low. There is a marked increase in the size of the cell and of its nucleus (fig. 4, E) and in some cells double nucleoli, suggestive of endomitosis, appear. In Stage 4 accumulation continues until almost the entire cell is filled with large aggregates of product (fig. 4, G). At this time small granules fill the axon (fig. 4, H). Apparently there is a physical change in the substance secreted and it is transported in this finely divided state. In the last stage examined the cells are in senescent state; there is a diminution in the amount of product in the cell and the axon is filled with granules (fig. 4, I). The early Stage 4 cells resemble most closely the group state in diapause brains.

The cells of Group 3 contain a stainable product during diapause (fig. 2, C). The granules are smaller than in Group 1, separate from one another in some parts of the cell and associated in aggregate masses in other parts. In the non-diapause larva these cells do not contain any product until Stage 3. The first minute granules to be detected are associated with the periphery of vacuoles as in early Group 1 cells, but later they enlarge and spread into the surrounding cytoplasm (fig. 5, C). During Stage 4 this material increases in abundance and the last stage granules are abundant in transit in the axons (fig. 5, D, E). In non-diapause brains aggregates do not form, the granules remaining small and separate.

The abaxonal zone of Group 4 cells stains a relatively uniform pale purple colour during diapause (fig. 2, D). At this time no particles or granules of product can be distinguished. In the non-diapause larva the first signs of secretory activity appear some hours after the cessation of feeding, in Stage 2, when faint purple patches of regular outline form in the abaxonal zone of the cell. The sequence of accumulation in Group 4 is simply one of increase in

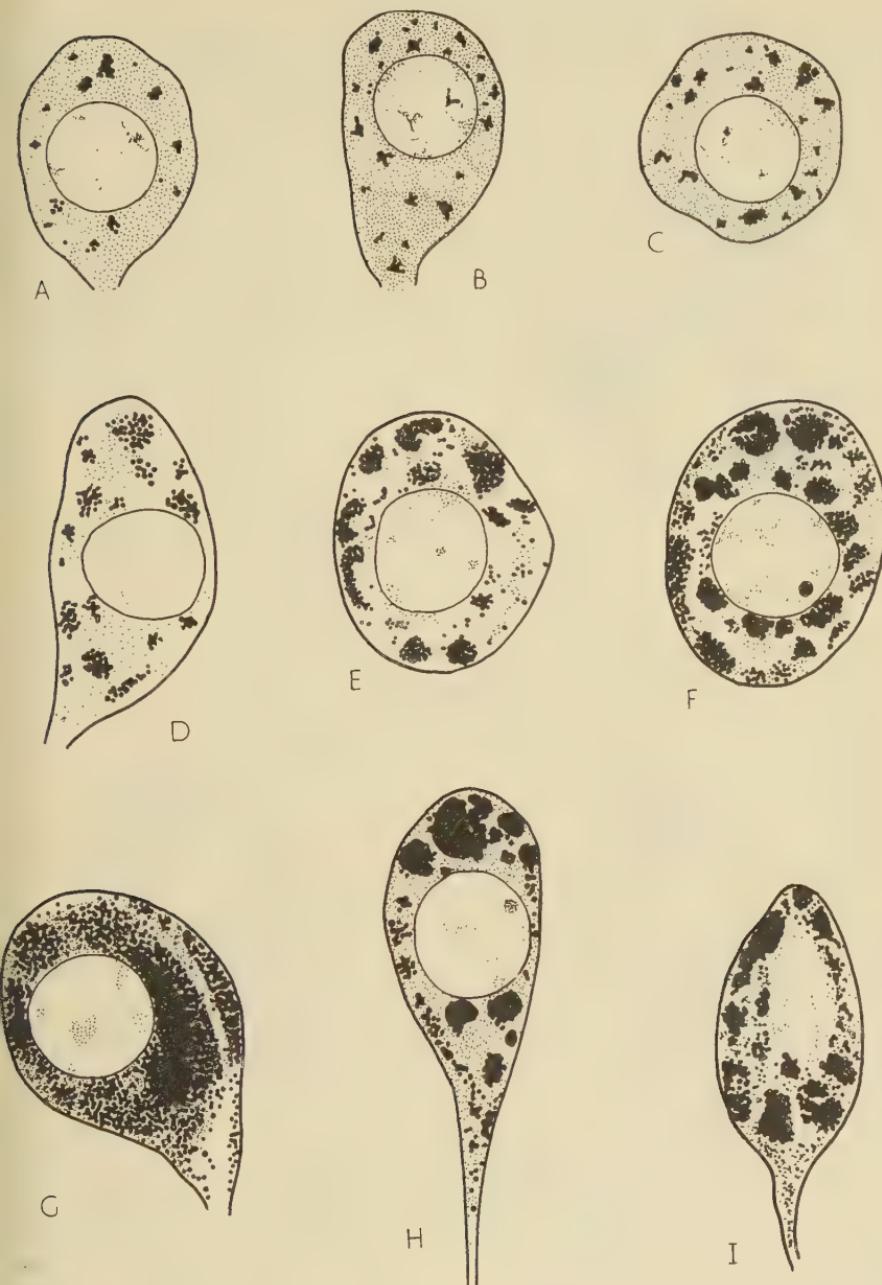


Fig. 4. Cells of Group 2; paraldehyde-fuchsin staining. A, Stage 1. B, early Stage 2. C, late Stage 2. D, early Stage 3. E, late Stage 3. F, early Stage 4. G, mid Stage 4. H, late Stage 4. I, Stage 5.

size and number of these 'blobs', which by late Stage 3 prove to be formed of minute purple granules. In Stage 3 the product tends to be associated with vacuoles, although this is not invariably so (fig. 6, A), and the vacuoles may be

entirely surrounded by a wide zone of secretory material. The highest degree of accumulation is attained and an almost total evacuation of the cell occurs in Stage 4; in the fifth stage the cells assume a faint uniform purple colour similar to that found in these cells during diapause. Pycnotic changes in the

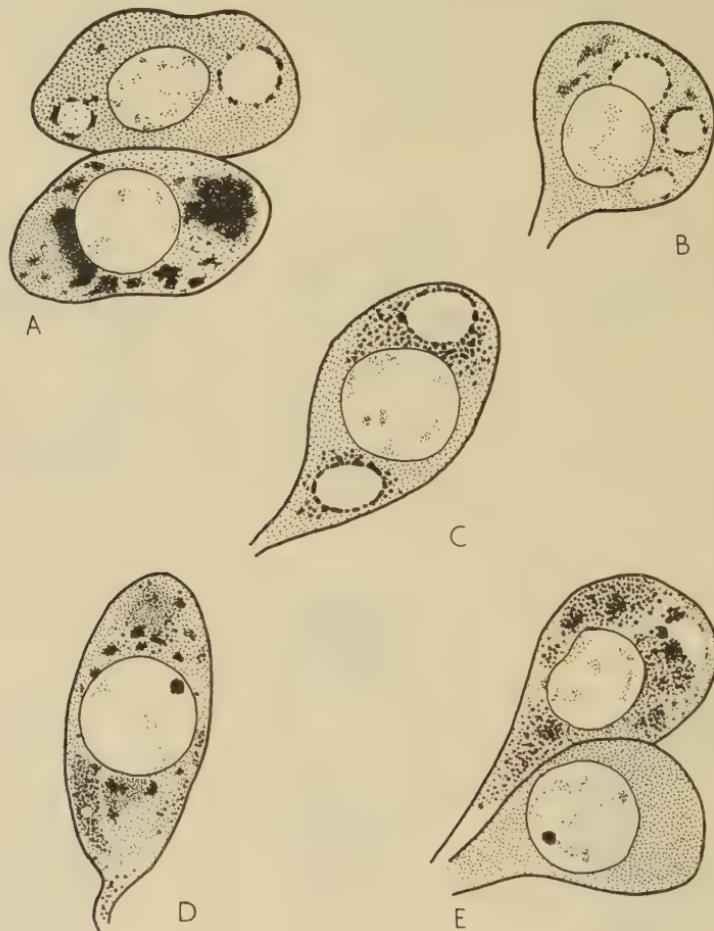


FIG. 5. Cells of Group 3; paraldehyde-fuchsin staining. A, Stage 3, the Group 3 cell lying above a Group 4 cell. B, Stage 3. C, Stage 4. D, Stage 5. E, Stage 5, the Group 3 cell lying above a Group 4 cell.

nucleus and other signs of senescence have not been observed up to this stage in development and it is possible that this group goes through another cycle of secretion later in the pupal instar.

The same uniform pale purple colour as was noted in Group 4 is produced by this stain in Group 5 cells during diapause (fig. 2, E). The cycle in the non-diapause larva is also similar to that in Group 4, the blobs appearing in Stage 3. These are succeeded by the appearance of fine granules in the cytoplasm

1 larger granules associated with vacuoles (fig. 7, A, B) but in Stage 4 the granules disappear and the cells revert to a uniform purple colour. The cycle starts later and is completed earlier than in Group 4.

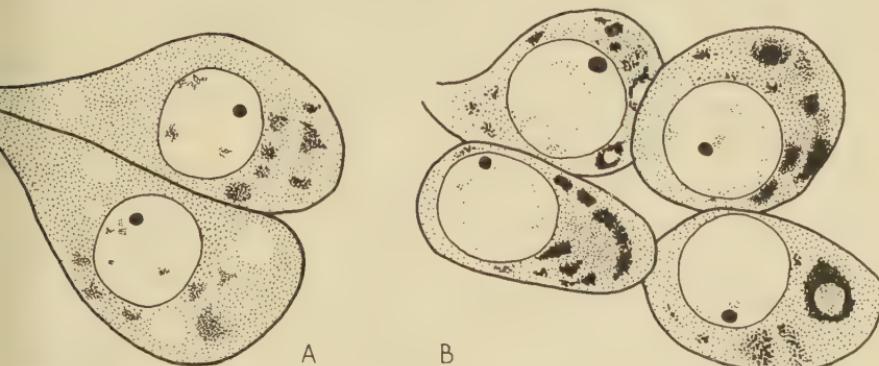


FIG. 6. Cells of Group 4; paraldehyde-fuchsin staining. A, Stage 2. B, Stage 4.

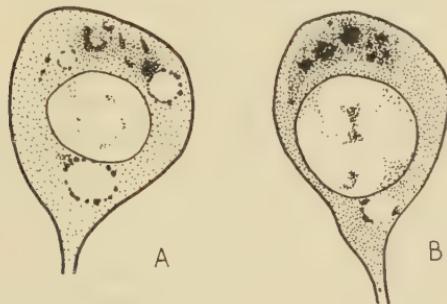


FIG. 7. Cells of Group 5; paraldehyde-fuchsin staining. A, Stage 3. B, late Stage 3.

Secretory granules are found in Group 6 cells in diapause. These are separate and do not match in size those of Group 1. In non-diapause brains a product is evident in these cells only in stage 3, when the appearance is similar to that in diapause brains (fig. 2, f). One specimen was sectioned at such an angle that the axon could be followed for a distance equal to twice the length of the cell (about  $40 \mu$ ) and its course could be traced directly to the nerve to the corpus allatum. It did not traverse the pars intercerebralis. The cells of Group 6 are therefore identifiable as 'lateral neurosecretory cells'. The fact that they can only be found in Stage 3 brains indicates a very rapid completion of the cycle of activity in prepupae.

*Other staining procedures and histochemical tests.* The staining characteristics of the cell groups in diapause brains are summarized in table 1; those in non-diapause brains in table 2 (see p. 393). Owing to the conflicting evidence discussed below, provided by the histochemical tests on diapause material, it was decided that little information of value was to be gained from applying any of these to non-diapause material and they were omitted. The following points are important.

All groups, except Group 6, contain granules staining a dark blue colour with chrome haematoxylin at their peak of activity in the prepupa, but only Group 3 cells contain such granules during diapause. In diapause the cytoplasm of Groups 4 and 5 is markedly acidophil and this is to some degree retained in the developing larva. At all stages this acidophil material is Group 4 mildly and in Group 5 strongly PAS-positive. During diapause sudanophilia is a property of the cytoplasm in Group 5, but in Group 2 it is a property of the granules of secretion.

#### *Neurosecretory cells of the brain at the termination of diapause*

Mitoses were observed in paraldehyde-fuchsin preparations of brains of several larvae which had been in diapause for 6 weeks. In these, it can be assumed, diapause had just terminated. Only Groups 4 and 5 differed from their diapause appearance. The colour of the cytoplasm was no longer uniform and the distribution of granules now resembled that in late Stage 3 non-diapause cells. It can be concluded that the activation of these groups coincides with the termination of diapause.

#### *The corpora in *L. caesar* larvae*

*Corpus cardiacum.* Secretory granules, stained by paraldehyde-fuchsin and by chrome-haematoxylin, occur in this organ during diapause. Results obtained with the former stain suggest that these could originate in Groups 1, 2, 3, or 6, but corresponding granules are only demonstrated by the latter stain in Group 3.

In the post-feeding non-diapause larva the first granules appear in the corpus cardiacum during Stage 2, just after the inception of activity in Groups 1 and 2. The concentration of this material increases in the successive prepupal stages. The product enters the organ in a finely granular state in the axons of the nerves from brain to corpus cardiacum. These axons penetrate between the cells of the corpus. Ovoid bodies form, which are swellings of nerve fibres caused by local accumulations of the secretory material (M. Thomsen, 1953). These swellings contain aggregates of larger granules than the type seen in transit. The material is abundant in the lumen of the aorta and presumably passes through the wall of the vessel into the blood. It has not been determined by histological methods whether the corpus cardiacum modifies the product before passing it into the blood, nor indeed is it known if the stainable product is the active principle or only a carrier substance. It is confirmed that the corpus cardiacum has an independent endocrine function.

*Corpus allatum.* There is no evidence of receipt of neurosecretory product by the corpus allatum at any of the stages examined. There is certainly a substance which stains strongly with paraldehyde-fuchsin in late Stage 4 and Stage 5 corpora allata, but this is associated with the tracheae of the organ. Caution must be exercised in interpreting the results obtained with that stain in insects about to moult, since tracheal chitin which has been attacked by moulting fluid and the chitin precursor in the tracks of new tracheae are strongly coloured by the purple stain.

## DISCUSSION

Six groups of neurosecretory cells have been distinguished in the brain of third instar larva of *L. caesar*. While a similarity in the chemical composition of the product of the various groups is indicated by a common affinity for aldehyde fuchsin after oxidation, there are clear cytochemical differences between the 6 groups, which were separated originally on physical differences, the mode of accumulation of the granules of product. The cytochemical tests were selected with the object of identifying the carbohydrate component of the product recorded by Gabe (1954). The following conclusions are based on the discussion of histochemical tests for carbohydrates and lipids in Mori (1952) and Pearse (1953).

In Group 1 the product has an affinity for alcian blue and is PAS-negative, results consistent with the presence of an acid mucopolysaccharide, but the metachromasia produced with toluidin blue does not support this conclusion. The cytoplasm of a Group 4 cell in diapause is eosinophil, PAS-positive, not Sudanophil and produces  $\beta$  metachromasia with toluidin blue. These results are consistent with the presence in the cytoplasm of a neutral mucopolysaccharide (or mucoprotein) not found in other neurones. The staining in Group 5 matches in some respects that of Group 4 but the PAS-positive reaction is stronger and the cytoplasm is strongly and uniformly Sudanophil. These results might indicate the presence in the cytoplasm of Group 5 of a glyco- or phospholipid or lipoprotein but the conclusion is again controverted by the metachromasia produced with toluidin blue. The results obtained with the other cell groups were, as can be seen in tables 1 and 2, equally inconclusive. There is no evidence that the product of these cells has a polysaccharide component, an observation in agreement with that of Wigglesworth (1956) and controverting that of Gabe (1954), who claimed that there is detectable in this product a 'glucide' component other than glycogen. In the two groups in which such a component occurs it is a factor in the cytoplasm and not of the product. After the completion of these investigations evidence was produced by Sloper (1957, 1958) that substances selectively stained by the paraldehyde fuchsin procedure contain protein-bound cystine. According to Saffran and others (1958) the vertebrate neurohypophysis contains a family of chemically related peptide hormones. A common component of these is probably cystine, which accounts for their staining characteristics. If the opinion of Sloper (1957), that the neurosecretory proteins of vertebrates and invertebrates are similar, is accepted, then it can be postulated that the products of the 6 cell groups in *L. caesar* larvae are proteins or peptides, all of which have at least one amino acid in common, cystine; but each must have a composition differing by one or more other amino-acids from its closest relative. This difference may account for the chemical dissimilarities between the groups which have not been demonstrated.

Chrome-haematoxylin cannot be used to demonstrate phases in the cycle of activity of a group, but the characteristic blue-stained granules appear at the

peak of activity of a cell and are found in the corpus cardiacum. It is concluded that when these granules appear in a group the cells are in an effective discharge phase of activity.

Wigglesworth (1934) first suggested that diapause might be due to a temporary failure in the secretion of the growth hormone. This postulation was confirmed in *Platysamia* by Williams (1946). Chilling for a certain period of time terminates pupal diapause in that species. The brain by means of a diffusible hormone liberated into the blood activates the thoracic gland whose secretion promotes pupal development. Thus the unchilled diapause brain can be regarded as 'inactive' and the chilled brain as 'active'. Results from experiments with *L. caesar* larvae (Fraser, 1957) indicate that the diapause brain is, in that species, similarly failing to activate the thoracic gland and is in this sense 'inactive'.

Since neurosecretory activity in larvae has hitherto only been related to the process of thoracic gland activation, it might be expected that the neurosecretory cells in the brains of *L. caesar* larvae in diapause would appear to be totally inactive and devoid of product, as in feeding larvae. On the contrary, abundant granules are demonstrable in Groups 1, 2, 3 and 6 and in the corpus cardiacum. The brain cells maintain a remarkably consistent appearance throughout diapause. It might therefore be suggested that the product accumulates in these cells shortly after the cessation of feeding, as in the non-diapausing larvae, but is not discharged until the state terminates. There is, however, a small amount of product in the corpus cardiacum which might be due to casual leakage from 'charged' cells in ineffective amounts. This is probably so in the case of Groups 1, 2, and 6, but Group 3 provides a clear discharge picture with chrome-haematoxylin, and after staining with paraldehyde-fuchsin closely resembles Group 4 at the peak of its activity in the non-diapausing larva. Group 3 is thus the probable source of most of the material in the corpus cardiacum and may be the only group with a physiological function during diapause. These cells, instead of going through a normal rapid cycle of accumulation and discharge lasting 3 or 4 days, reach a certain level of activity which is maintained for as many months as the diapause lasts. Neurosecretory cells capable of similar monotonous activity are to be found in the ventral ganglia (Fraser, 1957). The absence of a stainable product from Groups 4 and 5 during diapause is satisfactory evidence of their inactivity.

Since all the cell groups go through a cycle in the prepupa, it is clear that they are all concerned in the control of physiological processes associated with moulting and metamorphosis. The time of thoracic gland activation can be determined by a study of the growth of the imaginal discs, whose development, in the post-feeding pre-pupation phase, is promoted by the secretion of that gland. It has been found that the discharge of Group 4 cells corresponds most closely with the time of gland activation. Thus Group 4, inactive during diapause but becoming active at its cessation, appears to be the most probable source of the activating substance. Accumulation in Groups 1 and 2 certainly starts early in the post-feeding phase but it continues, with the consequent

tion of the cells, almost to the time of puparium formation, when there is rapid discharge of product. Group 3 does not approach a peak of activity until after puparium formation. Group 5, inactive in diapause like Group 4, becomes active later and completes its cycle of activity earlier in the prepupa. It therefore seems unlikely that Groups 1, 2, 3, or 5 are prime stimulators of the thoracic gland. In the developing prepupa Group 6 has a brief cycle of activity which is only evident after thoracic gland activation; but since Williams (1948) has proved that both medial and lateral cells participate in that process and since Group 6 cells are the only ones identifiable as belonging to the latter category, it is probable that their secretion plays a part, perhaps in the establishment of autonomy, in the stimulation of the gland.

With the development of a microcautery technique it may soon be possible to elucidate the physiological role of certain of these cell groups now that it has been established that the median neurosecretory cells are not a homogeneous group. Progress has been hampered in the past by the failure of the early investigators in this field to appreciate this fact. Descriptions of the secretory cycle which have previously been produced are often unsatisfactory, because the varied aspects of different groups have been equated with successive phases of the secretory cycle in a homogeneous group and the assumption has been made that various properties of the different groups are common to all neurosecretory cells.

At the cessation of feeding, whether the larva of *L. caesar* develops directly into diapause, the neurosecretory system is alerted and cells that have been distinguishable from common neurones by position and form only during the feeding phase, assume distinctive staining characteristics. This is particularly true for those groups which in both types of post-feeding larva have a granular product and for Groups 4 and 5 which assume in diapause the appearance interpreted as the resting state. The completion of feeding and the changes in neurosecretory cells are related processes. A relationship between feeding or nutrition and the hormone cycle has long been known in insects (Giles, 1933 and subsequent papers). It could be said that in a blowfly larva which stops feeding voluntarily, the same mechanism which terminates feeding activity also alerts the brain neurosecretory system; but if the crop of a non-diapause species such as *C. vomitoria* is removed prematurely from its food, the duration of the post-feeding pre-pupation period after this voluntary cessation is the same as that after voluntary cessation. Therefore the mechanism which normally causes the larva to stop feeding and that which alerts the brain cells must be separate. At the normal cessation of feeding the evacuation of the crop begins, as its contents are evacuated without replenishment. After this come the first signs of neurosecretory activity. The activation of the neurosecretory system may therefore be related to the event of crop evacuation. Lees (1955) favoured the theory that secretion of brain hormone is induced by nervous stimuli, but Monro (1956) has shown that in the larva of a noctuid moth the stimulus is humoral. Either theory is possible. It does not seem significant that the stomatogastric nervous system is linked to the corpus

cardiacum, which, in turn, has nerve connections to the brain. The fact that feeding has ceased may thus be recorded by the stomatogastric nervous system, perhaps as a result of release of tension in the crop, this system thus providing either a humoral stimulus liberated through the neurohaemal organs or a nervous stimulus transmitted directly to the brain. This stimulus, humoral or nervous, activates the neurosecretory system.

If environmental conditions are favourable during the time when crop retraction and activation of the neurosecretory system occur, in the *L. caesar* larva, the neurosecretory cycle is completed directly in all groups; but if conditions are unfavourable, activity halts in Group 4 and 5 before the formation of a particulate product and in Groups 1 and 2 when accumulation of product is complete; while Group 3, and perhaps Group 6, settle down to continuous production. Another mechanism has come into operation blocking the cycle in certain groups, the result being that the thoracic glands remain inactive during diapause. There is good evidence (Cragg and Cole, 1952) that the sensitivity of this mechanism is maternally determined.

In conclusion the author wishes to thank Drs. A. R. Hill and D. G. Cochran of the Department of Zoology, University of Glasgow, for their advice and encouragement; Dr. H. F. Steedman and Mr. J. B. Cowey (also of this department) for advice on staining techniques; and Mr. W. F. Smith for valuable technical assistance.

TABLE I

*Staining characteristics of brain neurosecretory cells in the larva of *L. caesar* during diapause*

<i>Staining procedure</i>	<i>Group 1</i>	<i>Group 2</i>	<i>Group 3</i>	<i>Group 4</i>	<i>Group 5</i>	<i>Group 6</i>
chrome-haematoxylin-phloxin	+	grey-blue	—	+	blue-black	uniformly phloxinophil
alcian blue neutral red	+	blue-green	—	—	uniformly red	uniformly red
alcian blue haemalum-eosin	+	—	—	—	uniformly eosinophil	uniformly eosinophil
Sudan black B	±	+	granules	±	±	++ uniform
toluidin blue	++	beta	+	beta	orthochromatic	± beta
PAS after diastase	—	—	—	—	+	++ uniform
acid diazonium	—	—	—	—	—	—

TABLE 2

Staining characteristics of brain neurosecretory cells in the larva of *L. caesar* during the prepupal phase

Staining procedure	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
me- hematoxylin- aloxin	++ blue-black	++ blue- black	++ blue-black	++ blue-black	++ blue-black	— blue- black
n blue hemalum- sin	++	—	—	—	—	—
after astase	—	—	—	± cytoplasm	+	cytoplasm

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# Neurosecretory Cells in the Abdominal Ganglia of Larvae of *Lucilia caesar* (Diptera)

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## SUMMARY

Ten neurosecretory cells can be found in each of the first 5 abdominal ganglia of the larva of *L. caesar* L. These are arranged as two lateral pairs and a single ventral cell in each half of a ganglion. Half the lateral cells go through a cycle of secretory activity before pupation in developing larvae. In diapause larvae while half the lateral cells are in a resting phase the remainder are in various stages of secretory activity. The destination of the product is not known. Chemically this product is, unlike that of brain neurosecretory cells, PAS-positive. It may contain a glyco- or phospholipid or lipoprotein component.

## INTRODUCTION

ACCOUNTS of neurosecretory cells in the ventral ganglia of insects are very rare in comparison with the numerous descriptions which exist of such cells in insect brains. They were first noted in the ventral ganglia of Lepidoptera by Day (1940), and were described in a cockroach by Scharrer (1941). Their occurrence has since been observed in the sub-oesophageal ganglia of Ephemeroptera (Arvy and Gabe, 1952 a, b), of Odonata (Arvy and Gabe, 1952c), of *Tenebrio* (Arvy and Gabe, 1953), of *Bombyx mori* (Bounhiol, Arvy and Gabe, 1953), and of *Iphita* (Hemiptera) by Nayar (1953). Lhoste (1953) indicated that the ganglia of the ventral nervous system of *Forficula* contained neurosecretory cells but he did not describe them. Wigglesworth (1955), discussing humoral stimuli concerned in the breakdown of the thoracic end of *Rhodnius*, suggested that 'perhaps the neurosecretory cells which are conspicuous in the thoracic and abdominal ganglia of *Rhodnius* and other insects may be concerned'.

Neurones are identified in this study as neuro-secretory cells by their morphological similarity to certain brain-cells of this kind, by their staining characteristics, and by the evidence in some of the axonal transport of secretory granules.

## MATERIAL AND METHODS

Serial sections were prepared from material fixed in Bouin, embedded in paraffin wax, and cut at 6  $\mu$ . Sections of the ganglia of larvae in diapause were stained with chrome-haematoxylin-phloxin (Gomori, 1941) and paraldehyde-phenol-hsin (Halmi, 1952), in both cases after oxidation with potassium permanganate. The histochemical tests employed were alcian blue with neutral red and haemalum-eosin counterstaining (Steedman, 1950); periodic acid / Schiff

(PAS) with and without pretreatment of sections with diastase (McManus, 1946); and toluidin blue for metachromasia (Pearse, 1953). McManus' Sudan black B test for lipids (Pearse, 1953) was applied to material fixed in formaldehyde-calcium solution. Ganglia of non-diapause prepupae, killed

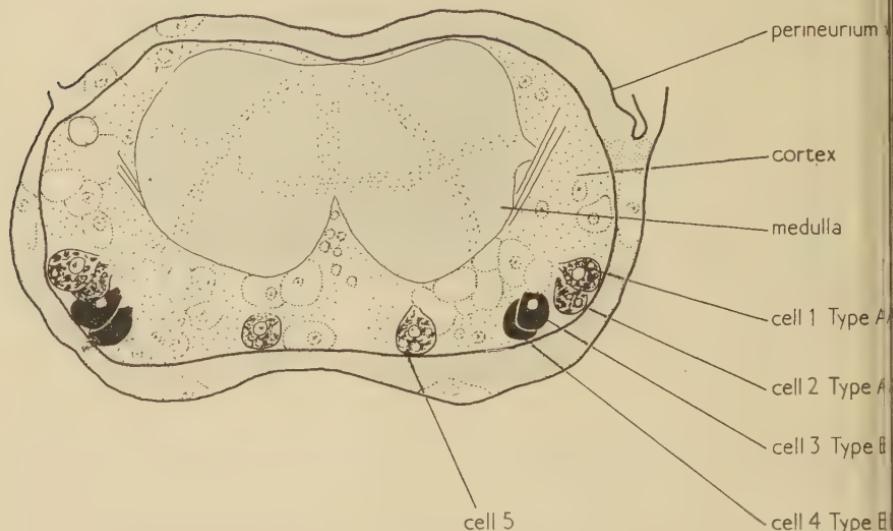


FIG. 1. Transverse section of the first abdominal ganglion of a larva of *L. caesar*, showing the distribution of neurosecretory cells. (From a camera lucida drawing.)

5 stages between the cessation of feeding and puparium formation, were sectioned and stained with paraldehyde-fuchsin and the PAS test was applied to these also.

## RESULTS

Neurosecretory cells could not be identified in the sub-oesophageal thoracic ganglia of *L. caesar* larvae. A few phloxinophil cells were seen in the sub-oesophageal ganglia of diapause larvae. These might have been in a resting state, like Group 4 cells in the brain (Fraser, 1957), but there was no evidence of a secretory function in the non-diapause prepupal ganglia.

Neurosecretory cells occur in abdominal ganglia 1 to 5. These are pyriform unipolar neurones measuring 20 to 25  $\mu$  long by 10 to 14  $\mu$  wide and have a central nucleus about 8  $\mu$  in diameter. These cells are symmetrically arranged in each ganglion (fig. 1). Four lie on each side at the ventrolateral angle of the ganglion (cells 1-4) and one on each side in a ventral position (cell 5). The four lateral cells can be separated into an upper posterior (cells 1 and 2) and a lower anterior pair (cells 3 and 4). Activity of the corresponding cells on each side is normally, though not invariably, in phase. The lateral cells in which granules of product are found are classified as Type A and those lacking such a product as Type B. Cells 1 and 2 are usually Type A and cells 3 and 4 Type B.

but any pair may be of the first type and, rarely, 3 of the 4 lateral cells are one type.

#### *Neurosecretory cells in abdominal ganglia of diapause larvae*

In sections stained with paraldehyde-fuchsin, secretory granules are found in Type A cells and in cell 5. In cell 5 the granules are rather small (up to  $\mu$ ), separate, and distinct, and the general facies resembles that of a brain-cell belonging to Group 6 (Fraser, 1957). In some Type A cells there is a high concentration of minute granules (about  $0.25 \mu$ ) while in others the granules are large (up to  $0.75 \mu$ ) and very distinct, but the total quantity of product appears to be less in the latter. The association of granules with vacuoles, a feature in most groups of brain neurosecretory cells, is also found in Type A cells. The cytoplasm of Type B cells is uncoloured by paraldehyde fuchsin and there are no positively stained inclusions. There are several large vacuoles in each cell.

After chrome-haematoxylin-phloxin staining the cytoplasm of Type B cells proves to be strongly and uniformly phloxinophil. In this they resemble brain group 4 cells in diapause. The Type A cells may contain very distinct blue-black granules; the rest of the cell, apart from the nucleus, is completely unstained. Alternatively, the granules may be minute and less easily discernible against a grey-purple ground cytoplasm.

The cells with large distinct granules detected by both staining procedures undoubtedly correspond. The ventral cell 5 is not differentiated by the second criterion; this is also true of brain Group 6 cells.

The secretory granules of Type A cells do not have a selective affinity for Janus blue, but they are PAS-positive. Pretreatment with diastase proves that the carbohydrate component is not glycogen. Sudanophil granules are usually found in two of the lateral neurones. These are certainly Type A cells. Sudanophil material occurs in variable amounts in other neurones but does not respond in form, concentration, or distribution to the granules found in Type A. With toluidin blue the Type A cells stain orthochromatically but the Type B cells exhibit metachromasia (beta): they stain a uniform violet colour. The cytoplasm of the B cells contains an appreciable concentration of a substance not found in any other neurones in the ganglia. This substance cannot be a mucoprotein or mucopolysaccharide since it is not PAS-positive. The results obtained with Type A cells are consistent (Pearse, 1953) with the presence in the granular product of a glyco- or phospholipid or lipoprotein component.

#### *Neurosecretory cells in the abdominal ganglia of non-diapause larvae*

The first signs of neurosecretory activity appear about 8 h after the cessation of feeding, in the first abdominal ganglion. With paraldehyde fuchsin staining a varying density of small granules can be seen in Type A cells; but Type B cells, identifiable by position and structure, are not selectively coloured. Within 3 days (at  $25^\circ \text{C}$ ) the full complement of 20 Type A cells can

be counted. These contain large separate granules, 0.5 to 0.75  $\mu$  in size. In the final stage of prepupal life, as the white pupa forms, the amount of product in the cells is diminished and the number of cells of this type has decreased on an average of 15. Type A cells have been found in these ganglia in positions 1 and 2, 1 and 3, and 3 and 4, the other two laterals being in each case Type B. Only 3 or 4 cells that contain positive granules have been found, per individual, in the cell 5 position.

The granules in Type A cells are again stained blue with chrome-haematoxylin while the cytoplasm of Type B cells is, as in diapause ganglia, uniformly phloxinophil. The histochemical tests indicate that the product is again sudanophil and strongly PAS-positive.

#### CONCLUSIONS

The axons of these cells take a circuitous route when they enter the medulla and it has not proved possible so far to trace an axon into its nerve. It is improbable that the destination of the granules, seen in axons in numerous sections, is a neurohaemal organ. The lateral and dorsal abdominal nerves and these ganglia all appear to terminate in the muscles of the body-wall and it seems possible, in the light of Finlayson's (1956) work on the abdominal muscles of Lepidoptera, that the secretion of these cells plays some part in the degeneration and regeneration of these muscles during metamorphosis.

The two variants of Type A cells noted in diapause larvae might be functionally different cells, but as the non-diapause material shows, they probably represent different phases in the secretory cycle of similar cells. The Type B cell may represent either a resting phase in the cycle of a Type A cell or a functionally different kind of cell, which is inactive in both types of prepupae but due to function later in the individual's life, or active but producing non-granular or colloid phloxinophil secretion. If the author's interpretation of the state of Group 4 brain cells during diapause (Fraser, 1957) is accepted, then it can be concluded that the Type B cell is in the resting state. The fact that any one of the 4 lateral cells, each of which has a fixed and definite location in the ganglion, may belong to either category indicates that A and B do in fact represent two aspects of a single type of cell.

Those cells which are active in the ganglia of non-diapause prepupae go through a cycle of activity which appears to be synchronous with the prepupal phase of activity in brain neurosecretory cells. The cells which are active during diapause produce a secretion which is chemically similar to that found in developing prepupae. They appear to be capable of monotonous activity but this is not sustained for the duration of diapause by all cells. Hence during diapause any one of the 4 lateral cells may be found in the resting state or may correspond in appearance with the waxing or waning phases of activity of cells in non-diapause prepupae.

Gabe's (1954) general statement that the neurosecretory product of insects has a carbohydrate component other than glycogen does not hold in the case of brain cells but is true for those in the abdominal ganglia, which are the only

urosecretory cells found in the *L. caesar* larva whose secretion is PAS-positive.

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# The Connective Tissue Sheath of the Locust Nervous System: A Histochemical Study

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## SUMMARY

The connective tissue sheath surrounding the nervous system of *Locusta migratoria* has been studied histochemically. It consists of an outer non-cellular layer, the neural lamella, and an inner layer of cells, the sheath-cells.

The neural lamella has been identified as being composed of a collagen-type protein and neutral mucopolysaccharide on the evidence of its histochemical reactions and the identification of hydroxyproline by paper chromatography in a hydrolysate of the neural lamella.

The sheath-cells possess large numbers of lipochondria composed of phospholipids and cerebrosides, and small spherical mitochondria. The cytoplasm also contains lipids (some of which may be cerebrosides), glycogen, and RNA.

## INTRODUCTION

SODIUM and potassium ions are always found in the body fluids of animals, a much higher concentration of sodium than potassium ions normally being present. Some insects, however, are peculiar in having very low concentrations of sodium ions, but high concentrations of potassium ions; in certain cases, less sodium than potassium may be present. Boné (1944) correlated the relative amounts of sodium and potassium ions in insect haemolymph with the feeding habits of the insect. He concluded that herbivorous insects have a very low sodium and a high potassium concentration, whereas omnivorous and carnivorous insects have the more usual higher sodium concentration.

These unusual sodium and potassium ratios might be expected to affect various physiological processes and especially those concerned with conduction in the nerves and muscles. The normal concentration of potassium ions in some insects is higher than that which would block conduction in crustacean or vertebrate nerves and this, together with the very low sodium concentration, led to the suggestion that conduction in insects involves a mechanism different from that in other animals (Hodgkin, 1951). Hoyle (1952, 1953) found that potassium concentrations of 70 mM had no effect on the action potential of the nerves of *Locusta migratoria*, but if saline containing 40 mM of potassium was injected under the sheath which surrounds the nervous system, conduction was blocked. From these observations it was concluded that the connective tissue sheath acts as a barrier, selectively permeable to ions, maintaining a constant ionic environment around the neurones and their axons; therefore, the nerves are not affected by the ionic concentrations in the haemolymph.

The sheath in the locust consists of two layers, an outer homogeneous non-cellular layer, with an inner layer of flattened cells (Hoyle, 1952). Little is known of the chemical nature of the outer layer, nor of the underlying cells in the locust, but there is evidence that the outer layer of some other insects may contain collagen and some mucopolysaccharides (Baccetti, 1955, 1956, 1957; Richards and Schneider, 1958). This paper is an account of a histochemical study of this sheath in *L. migratoria*.

### NOMENCLATURE

Several different terms have been used for the two layers of the sheath. The outer, non-cellular layer has most commonly been called the neural lamella (Schneider, 1902; Scharrer, 1939; Wigglesworth, 1950; Hoyle, 1952; Imms, 1957; Hess, 1958), but it has also been referred to as the perilemma (Wigglesworth, 1956; Smith and Wigglesworth, 1959) and the 'guaina neurale' (Baccetti, 1955). A similar situation exists for the underlying cells, since they have been called either the perineurium (Schneider, 1902; Scharrer, 1939; Wigglesworth, 1950, 1956; Baccetti, 1955), the epineurium (Imms, 1957), or the perilemma (Hoyle, 1952; Hess, 1958). The two layers together have been called the perilemma (Scharrer, 1939; Baccetti, 1955) and the epineurium (Imms, 1925), while the developing sheath has been called the neurilemma (Eastham, 1930; Roonwal, 1937; Johannsen and Butt, 1941).

In this paper it is proposed to use the term 'neural lamella' for the outer non-cellular layer. The underlying layer of cells will be referred to simply as the sheath-cells. It is suggested that the terms 'perineurium' and 'epineurium', which are used for different connective tissue layers in vertebrate nerves, should not be used, since the insect nerve-sheath is not homologous with that found in vertebrates.

### METHODS

The connective tissue sheath has been investigated in the metathoracic ganglion and the abdominal region of the nerve cord in *L. migratoria*. The locusts were kindly supplied by the Anti-Locust Research Centre, London.

The ganglia and nerve-cords were fixed in a variety of fixatives and then embedded in either paraffin wax, celloidin, or gelatin. The sectioned material was stained by a routine histological method or subjected to histochemical tests. The details of the procedures used appear in the appendix. Some living material was examined after staining with vital dyes.

The amino-acid composition of the neural lamella was analysed by paper chromatography. The neural lamella of the meso- and meta-thoracic ganglia was separated from the underlying cells; histological examination showed that the neural lamella could not be dissected entirely free from cells, but the cells dissected away were free from contamination by the neural lamella. As a control, therefore, the amino-acids of the cells were also analysed and the two analyses compared. After the cells and neural lamellae of about 45 ganglia had been separated, they were hydrolysed in 6 N hydrochloric acid.

00° C for approximately 16 h. The hydrolysates were evaporated to dryness under reduced pressure, redissolved in 10% iso-propyl alcohol, and then applied to No. 1 Whatman chromatography paper. The chromatogram was 2-dimensional; the first solvent being a mixture of butanol, acetic acid and water (40-10-50) and the second phenol saturated with water. The chromatograms were sprayed with a mixture of isatin and ninhydrin in butanol (after Lor and Roberts, 1957) to make the amino-acid spots visible.

## RESULTS

### *Structure of the connective tissue sheath*

Hoyle (1952) describes the sheath as consisting of two layers, the outer neural lamella and the inner sheath-cells, with a tracheolated membrane on the outer surface of the neural lamella. The two layers of the sheath are clearly visible in sections, but a tracheolated membrane has not been observed. There are, however, tracheae forming a network over the surface of the ganglia and nerves, but this network is not a continuous membrane surrounding the nervous system.

The neural lamella is about 7  $\mu$  thick around the metathoracic ganglion and 3  $\mu$  round the ventral nerve-cord. It is clearly differentiated from the sheath by trichrome staining methods. It is non-cellular and homogeneous; its structure was visible in the light microscope with the methods used in this study.

The sheath-cells (figs. 1 and 2) form a continuous layer under the neural lamella, about 4 to 15  $\mu$  thick. The cell boundaries are not clear as the cells are irregularly shaped. The nuclei are approximately 8  $\mu$  in diameter. The cytoplasm contains many lipochondria of different sizes, ranging from 1  $\mu$  to 5  $\mu$  in diameter. They have a tendency to congregate in the region of the cells adjacent to the neural lamella. The mitochondria can be seen after vital staining with Janus green and in preparations made by Baker's (1957) HPO technique. They are all spherical bodies, about 0.5  $\mu$  in diameter. They are distributed in all regions of the cells, but are not very numerous.

### *biochemistry of the neural lamella*

*Carbohydrates.* Some sections were tested with the periodic acid / Schiff (PAS) test which indicates the presence of carbohydrate groupings. The neural lamella is strongly positive. The reaction is not reduced by previous incubation in saliva; this indicates that glycogen is not causing the reaction. Incubation with hyaluronidase does not affect the result; it seems, then, that hyaluronic acid is not the cause of the positive PAS reaction. The neural lamella is PAS negative if the periodic acid treatment is omitted; therefore the positive reaction is not due to free aldehydes present in the tissue. These results suggest that a mucopolysaccharide is present in the neural lamella.

To find out if any acid mucopolysaccharide is present, some sections were treated with toluidine blue, because acid mucopolysaccharides are

metachromatic. But metachromasy does not occur in the neural lamella as so acid mucopolysaccharides are probably not present. After treatment with concentrated sulphuric acid (Lison, 1953), the neural lamella becomes intensely metachromatic and this suggests that most of the mucopolysaccharide present must be neutral.

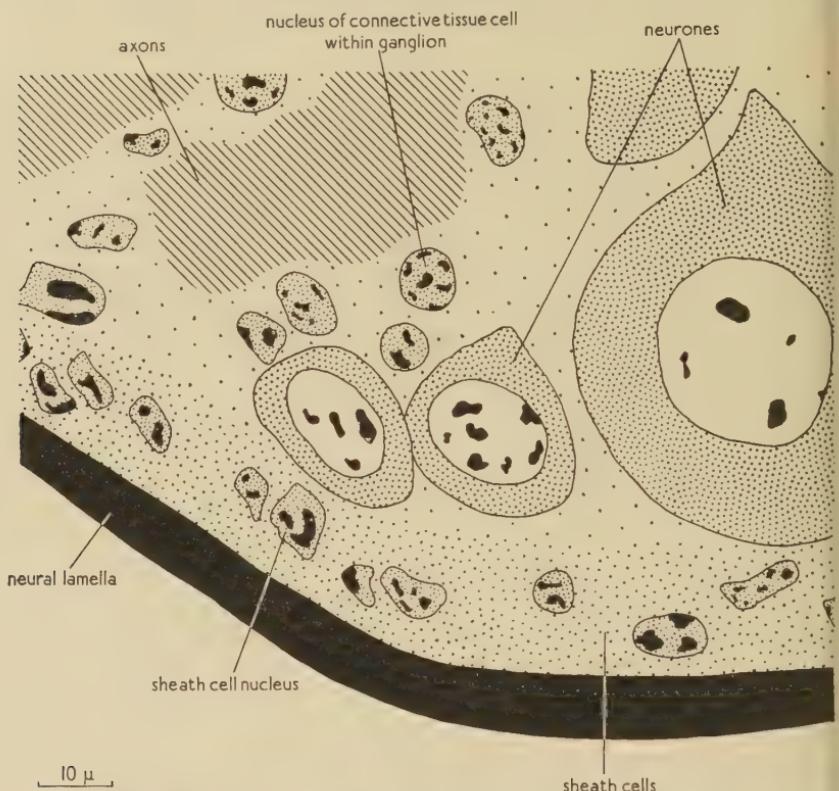


FIG. 1. A diagrammatic section of part of the metathoracic ganglion of *Locusta migratoria*.

This was further checked by estimating the basiphilia of the neural lamella by means of the methylene blue extinction test (Pearse, 1953); the ability to bind methylene blue at low pH, i.e. pH 2, indicates the presence of acid mucopolysaccharides or nucleic acids. In this case, the neural lamella does not stain with methylene blue below pH 5, and this again suggests that very little, if any, acid mucopolysaccharide is present.

*Lipids.* The neural lamella is not coloured by Sudan black B, nor does it give a positive result with the acid haematein test for phospholipids (Baker, 1946). Recent work has indicated that some lipids may not be in a detectable form after fixation in formaldehyde-calcium, since more lipids can be demonstrated in cells which have been fixed in a fixative containing chromic acid (Bradbury and Clayton, 1958). Several procedures for 'masked lipids', which

be described in greater detail later, were used, but still no lipid could be detected in the neural lamella. Therefore, if the neural lamella does contain lipid, it must be in amounts too small to be detected by these histological techniques.

**Proteins.** The cytochemical reaction for proteins described by Barnard and Nelli (1956), involving the coupling of proteins with a diazonium com-

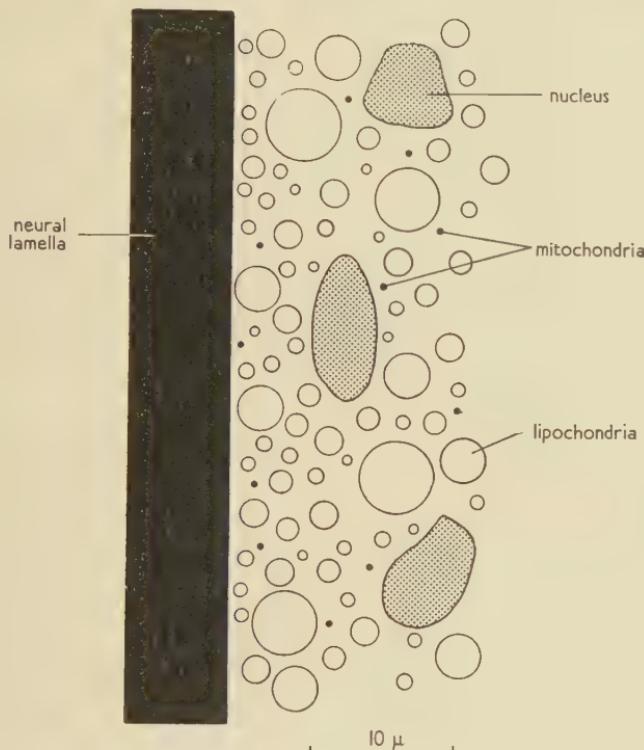


FIG. 2. A diagram to show the neural lamella and the cytoplasmic inclusions of the sheath-cells

nd, gives a positive result with sections of the neural lamella. This coupling reaction may be prevented by prior treatment of the sections with benzoyl oxide, except in the nuclei and in other special cases, for example, collagen (Barnard, personal communication), where it is not blocked by benzoylation. It was, therefore, interesting to find that the neural lamella is still positive to the coupling reaction after benzoylation. It may be mentioned here that the neural lamella is also positive to the PAS test after benzoylation, another characteristic of collagen (Barnard, personal communication).

In addition, the neural lamella gives a positive result with Baker's (1947) modification of the Sakaguchi reaction for arginine and other guanidine derivatives, which indicates that arginine is present. The results with the / nitrite test for phenols, especially tyrosine (Baker, 1956), were negative

and suggest that there can only be a small amount of tyrosine in the neural lamella.

*Conclusions.* The results of the histochemical tests for proteins suggest that a collagen-type protein may be present in the neural lamella. This possibility is supported by the results with the coupling reaction and also by the presence of arginine and the absence of large amounts of tyrosine in the neural lamella. Baker (1956) states that collagen is negative with the Hg / nitrite test and, furthermore, amino-acid analyses of various collagens show that it is usual for collagenous proteins to contain much more arginine than tyrosine (Randall, 1953).

Collagen is also associated with mucopolysaccharide (Jackson, 1954), but it is not yet certain how the protein and polysaccharide groups are linked together. As a result, collagen is found to be PAS-positive (Pearse, 1951) and it is interesting that the neural lamella also is PAS-positive. Furthermore, collagen does not contain appreciable quantities of lipid; nor does the neural lamella. These results, therefore, indicate that the neural lamella may possess a collagen-type protein with associated neutral mucopolysaccharide.

### *Histochemistry of the sheath-cells*

*Carbohydrates.* The cytoplasm is positive to the PAS reaction, the lipochondria appearing more strongly positive than the rest of the cytoplasm. The reaction is reduced if the sections are first treated with saliva; glycogen is therefore present. The reaction is negative if the periodic acid treatment is omitted.

*Lipids.* The lipids of the sheath-cells are rather complex in their distribution. In Sudan black preparations, there is a very dark coloration of the sheath-cells. It can, however, be seen that the lipochondria are very darkly coloured whilst the cytoplasm is a much lighter colour. The same is true after colouration with Sudan IV.

The lipochondria are strongly positive to the acid haematein test (Baker, 1946), but the cytoplasm is negative and may still be coloured by Sudan II. This suggests that the lipochondria contain phospholipids and that the cytoplasm must possess other lipids. That the positive reaction in the lipochondria is due to the presence of phospholipids is confirmed by the negative result after the pyridine extraction test. In the neurones, phospholipids in the lipochondria are associated with cerebrosides (Shafiq and Casselman, 1954), so it seemed possible that a similar combination may occur in the sheath-cells. To investigate this possibility some ganglia were fixed in either hot or cold acetone and then tested for the presence of lipids with Sudan black. After cold acetone fixation, both the cytoplasm and lipochondria are still coloured by Sudan black, but after hot acetone, the cytoplasm is negative and the lipochondria only very faintly coloured. This suggests that cerebrosides are present both the lipochondria and cytoplasm, since they are soluble in hot, but not cold acetone (Casselman and Baker, 1955). These lipochondria may be called

phos globules', a name suggested by Baker (1957) for lipochondria containing cerebrosides and phospholipids.

There is recent evidence (Bradbury and Clayton, 1958) that after fixatives containing chromic acid, e.g. Flemming's fluid, it is possible to detect more lipids in cells. This fixative was used and followed by the Sudan black and haematein tests, but no further lipid material could be detected.

*Proteins.* The cytoplasmic inclusions cannot be differentiated from the nuclei of the cytoplasm after the protein tests. The cytoplasm and nuclei are positive with the coupling reaction, but after benzoylation the reaction is positive only in the nuclei. The Sakaguchi test for arginine (Baker, 1947) and the Hg / nitrite test for tyrosine (Baker, 1956) are also positive in both the nuclei and the cytoplasm.

*Nucleic acids.* The nuclei of the sheath-cells are Feulgen-positive after hydrolysis with dilute hydrochloric acid, so it may be concluded that they contain deoxyribose nucleic acid. The cytoplasm is strongly basophil; this was shown by the pyronin / methyl green technique (Jordan and Baker, 1952). That the basophilia is due mainly to the presence of ribonucleic acid in the cytoplasm was shown by treating the sections first with ribonuclease (Bradbury, 1956); the coloration with pyronin was then very much reduced.

*Phosphatases.* The tests for acid and alkaline phosphatases (Gomori, 1952) gave negative results in the sheath-cells.

#### *Chromatography*

Hydroxyproline is generally supposed to occur in large amounts in both invertebrate and vertebrate collagens, but only in small amounts elsewhere. Hence, if it is found that hydroxyproline is abundant in the neural lamella, it is reasonable to deduce that some collagen is present.

Chromatograms of both the neural lamella and the cell hydrolysates were developed as described earlier. The positions of the separated amino-acids were demonstrated by spraying the chromatograms with an isatin and ninhydrin mixture, which has a greater specificity for hydroxyproline than either reagent alone (Kolor and Roberts, 1957). The chromatogram of the neural lamella hydrolysate showed a distinct hydroxyproline spot, which was identified by running an authentic sample of hydroxyproline on the same paper. The cell hydrolysate gave a very faint hydroxyproline spot, but 150 applications of the hydrolysate were put on the paper, whereas only 30 applications of the neural lamella hydrolysate gave a very distinct spot. (Equal volumes of hydrolysates in iso-propyl alcohol were obtained at the beginning of the experiment.) The much greater amount of hydroxyproline in the neural lamella hydrolysate must be due mainly to the neural lamella and not to the surrounding cells. The presence of small amounts of hydroxyproline in the cell hydrolysate suggests that small amounts of collagen may be present in the connective tissue within the ganglion.

The results of the amino-acid analyses, therefore, provide further evidence

for the presence in the neural lamella of collagen-type protein, since appreciable amounts of hydroxyproline are found only in the neural lamella hydrosate.

#### DISCUSSION

The possibility that the neural lamella may be composed of collagen fibres with associated mucopolysaccharide has been mentioned previously. This is inferred from the histochemical evidence for the presence of proteins and mucopolysaccharide in the neural lamella and also from chromatography showing the presence of the amino-acid, hydroxyproline.

The mucopolysaccharide is thought to bind the collagen fibrils together, but it is not yet clear whether the protein and polysaccharide are chemically linked or merely in association with each other. In developing collagen acid mucopolysaccharides are present; these have been identified as chondroitin sulphate and hyaluronic acid and it is thought that they serve to stabilize the collagen fibrils (Jackson, 1954). However, in the neural lamella there is no detectable acid mucopolysaccharide. This fact does not exclude the presence of collagen, since Williams (1957) found that the metachromatic properties of the ground substance of mammalian collagen are reduced as it matures, and Jackson (1957) found less sulphated mucopolysaccharides in mature collagen. Neutral mucopolysaccharides are found in other collagen and Consden and Brown (quoted by Ward, 1958) suggest that since neutral mucopolysaccharides are not so readily removed as some other mucopolysaccharides, their association with the collagen must be very intimate. The neural lamella is then almost certainly composed of a collagen-type protein in association with an unknown mucopolysaccharide.

The polysaccharide content of collagens is variable, but it is thought that a high content of polysaccharides confers a greater degree of plasticity to connective tissue fibres (Bradfield, 1950). Although no estimations of polysaccharide content have been made in this case, the results suggest that a considerable amount is present. This would seem to agree with the mechanical functions of the neural lamella which are to hold together the cells and axons of the nervous system and yet be flexible enough not to resist or impede movements of the body.

It has generally been assumed that the neural lamella is secreted by sheath-cells (Scharrer, 1939). There appears to be no direct evidence for this. The origin of the sheath in the locust embryo is said to be from outlying ganglion cells which form a layer of cells around the ganglion (Roon, 1937). The formation of the neural lamella is not mentioned in this study, but it probably develops at a stage later than has been studied. If it is secreted by the sheath-cells, one might expect to find evidence for this in the enzymic content of the cells: Bradfield (1946) suggested that alkaline phosphatases are associated in insects with cells concerned with the synthesis of fibrous proteins. But both acid and alkaline phosphatases appear to be absent from these cells, at least in the adult locusts used in this study. It must be

ed that Day (1949) found alkaline phosphatases in the cerebral ganglion, not in the ventral nerve-cord of adult *L. migratoria*.

The identification of collagen in the locust is of special interest since there is very little reliable evidence for its presence in insect connective tissues. Rudall (1955) has identified collagen in the ventral nerve-cord of adults. Baccetti (1955) in a histochemical study of the sheath round the nervous system of *Anacridium aegyptium*, rejected the possibility that collagen was present in the neural lamella on his data, but later (Baccetti, 1956, 1957), in studies of the birefringent properties, he identified a collagen-type protein in the neural lamella of this insect. He found that the neural lamella of *A. aegyptium* could be divided into three regions; the narrow inner and outer regions being differentiated from the middle region by possessing neutral mucopolysaccharide, while the middle region has acid mucopolysaccharide. No such zonation of the neural lamella can be seen in the locust, and no acid mucopolysaccharide is present. The cockroach, also, has a sheath similar to that of the locust (Twarog and Roeder, 1956); the neural lamella possesses occasional nuclei which are thought to represent fibroblasts. This neural lamella was shown to be collagenous by Richards and Heider (1958). Wigglesworth (1956) found that the neural lamella was  $\alpha$ -positive in *Rhodnius prolixus*, which suggests a possible similarity with the locust's neural lamella. Moreover, electron micrographs of the neural lamellae of both the cockroach (Hess, 1958) and *R. prolixus* (Smith and Wigglesworth, 1959) show fibres with a periodicity similar to that of vertebrate collagen. A similar type of connective tissue sheath, the perineurium, consisting of an outer layer with collagen fibres and an inner epithelial layer, is present round vertebrate nerves.

In addition to the structural similarity between the sheath in insects and the perineurium in vertebrates, there appears to be also a similar function. There is evidence that the perineurium regulates the passage of ions into the peripheral nerves (Feng and Liu, 1949; Huxley and Stämpfli, 1951; Krnjević, 1954). But as Twarog and Roeder (1956) point out, the locust sheath is far more efficient than the vertebrate or cockroach sheath, since nerves in these insects are rapidly blocked by saline containing 50 mM of potassium (Roeder, 1948), while 140 mM of potassium takes many hours to block the locust nerve. The ionic regulation is in both directions across the sheath, and this explains why a block takes so long to occur in a sodium-free medium. The outer layer of cells and not the neural lamella seems to be responsible for the ionic regulation, since Krnjević (1954) and Twarog and Roeder (1956) found that silver nitrate penetrating the sheath of frogs or cockroaches was accumulated in the sheath-cells and did not go farther into the ganglion. Hoyle (1953) discovered that there was no ionic regulation in the locust if the neural supply to the nervous system was severed; an observation which suggests that regulation is an active process and hence would be more likely to occur in the sheath-cells than in the neural lamella. It has also been suggested by these authors that the sheath has an osmo-regulatory function.

It may be mentioned that Edwards, Ruska, and de Harven (1958), in electron microscope study of wasp peripheral nerves, in which they identify the neural lamella as the basement membrane of the sheath-cells, or lemniscate, suggest that this basement membrane may serve to maintain a constant ionic concentration at the plasma membrane of the cells, whilst the plasma membrane is the selective ion barrier. If this is the correct sequence of events the mucopolysaccharides of the neural lamella may be responsible for controlling the ionic concentration; there is recent evidence that mucopolysaccharides might be concerned in the control of the passage of ions across tissues (Bradbury, personal communication; Hess, 1955; Kantor and Schubert, 1957).

The connective tissue sheath seems to have, therefore, two functions: the neural lamella encloses the nervous system and restricts it mechanically, possibly controls the flow of ions across it, whilst the cells form a selective barrier to ions entering the nervous system. There is no apparent structural difference to account for the greater efficiency of the locust's sheath compared to that of other animals. Perhaps the difference is due to the fact that the locust has under normal conditions to tolerate fluctuations in potassium concentration not found in animals other than herbivorous insects (Hoyle, 1957).

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## APPENDIX

## Table of methods and results

KEY: + + + = strong reaction. + + = medium reaction.  
 + = weak reaction. — = no reaction.

Test or technique	Reference	Sheath-cells	Neural lamella
Tasson	Pantin, 1948	red	green
Mallory's collagen stain	Mallory and Wright, 1924	yellow	blue
Mallory's trichrome	Mallory and Wright, 1924	red	blue
IPO	Baker, 1957	mitochondria black	colourless

## APPENDIX (cont.)

Test or technique	Reference	Sheath-cells	Neural lame
<i>Carbohydrates</i>			
PAS	Pearse, 1953	++	+++
PAS with no oxidation		—	—
PAS after saliva		+	+++
PAS after hyaluronidase		++	+++
PAS after benzoylation		—	+
Toluidine blue	Baker, unpublished	—	—
Toluidine blue after sulphuric acid	Lison, 1953	++	++
Methylene blue extinction	Pearse, 1953	below pH 2.6	about pH 5
<i>Lipids</i>			
Sudan IV	Herxheimer, 1901	lipochondria +++ cytoplasm ++	—
Sudan black	Baker, 1945, 1949, 1956	lipochondria +++ cytoplasm ++	—
Acid haematein	Baker, 1946	lipochondria +++ cytoplasm —	—
Acid haematein: pyridine extraction	Baker, 1946	—	—
Sudan black after cold acetone	Casselman and Baker, 1955	lipochondria +++ cytoplasm ++	—
Sudan black after hot acetone	Casselman and Baker, 1955	lipochondria + cytoplasm —	—
Nile blue	Cain, 1947	lipochondria blue cytoplasm blue	—
Leibermann	Lison, 1953	—	—
<i>Proteins</i>			
Coupling reaction	Barnard and Danielli, 1956	++ +	+++
Coupling reaction after benzoylation	Barnard and Danielli, 1956	—	+++
Sakaguchi	Baker, 1947	++	++
Hg / nitrite	Baker, 1956	++	—
<i>Nucleic acids</i>			
Feulgen	Feulgen and Rossenbeck, 1924	nucleus ++	—
Feulgen control		—	—
Pyronin / methyl green	Jordan and Baker, 1955	++	++
Pyronin / methyl green after RNAase	Bradbury, 1956	+	++
<i>Phosphatases</i>			
Alkaline phosphatase	Gomori, 1952	—	—
Acid phosphatase	Gomori, 1952	—	—

# Histochemical Studies of *Herpetomonas muscarum*

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## SUMMARY

The cytoplasm of the 'leptomonas form' of *Herpetomonas muscarum* contains a parabasal body, mitochondria, and so-called 'volutin' granules. The parabasal body consists of neutral lipid (probably triglyceride), lipoprotein, and DNA. The mitochondria are in the form of granules. The volutin granules are more numerous and smaller than the mitochondria. The volutin granules appear to be composed of ribonucleoprotein. Glycogen is dispersed homogeneously in the cytoplasm. The cytoplasm is rich in RNA and protein. The nucleus is DNA-positive. Near the parabasal body lies a basal granule; a long flagellum arises from it. A flagellar vacuole lies near the base of the flagellum.

## INTRODUCTION

MANY genera of Trypanosomatidae (Flagellata) have been investigated previously by both light and electron microscopy.

Duboscq and Grassé (1933) homologized the parabasal body of the flagellates, consisting of chromophil and chromophobe parts, with the 'Golgi apparatus'.

Berge (1942, 1946) described small 'volutin' granules composed of ribonucleoprotein in *Trypanosome gambiense* and *T. evansi*. Anderson and others (1956) described similar volutin granules in *T. equiperdum*. Anderson and others (1956) failed to identify mitochondria in this species by electron microscopy.

Causey (1927) regarded the parabasal body of *Leishmania brasiliensis* as a derivative of the mitochondria. Sen Gupta and others (1953) described neutral red vacuoles, mitochondria, volutin granules, and kinetoplast as separate cytoplasmic inclusions in the flagellate form of *L. donovani*. The kinetoplast included a parabasal body, flagellar vacuole, and base of flagellum with a terminal blepharoplast. Chang (1956), using the electron microscope, described in *L. donovani* fine granules, vacuoles of various sizes, chromophil lipid droplets, and certain other bodies; the latter, according to him, might be mitochondria, but their internal structure was not clear. Guha and others (1956) observed only bigger and smaller granules in this species, and identified all of them as mitochondria.

Fantham (1912) described a flagellum arising from the kinetoplasm in *Herpetomonas pediculi*. Wilson (1925) and Wenyon (1926) described in *Herpetomonas* sp. a 'cone of fibrillae' connecting the parabasal body with the blepharoplast. Grassé (1926) regarded a clear space surrounding the 'kinetoplasm' or parabasal apparatus of *Herpetomonas* as the chromophobe substance. Khajuria (1950) claimed that small granules, representing the 'presubstance' of the true Golgi material (Hirsch, 1939), were budded off from the undifferentiated

tiated lipid parabasal body of *H. muscarum*; they could be stained with neutral red. He denied the presence of mitochondria in this species.

Prowazek (1904), Chatton and Leger (1911), and Kofoid and McCullough (1916) described axial filaments or fibres in the cytoplasm of some species of *Herpetomonas*. Kleinschmidt and Kinder (1950) described a fibrillar structure in the periplast in *T. lewisi* and *T. brucei*; Kraneveld and others (1951) described nearly parallel, longitudinal fibrils in *T. evansi*; Meyer and Pons (1954) described sub-pellicle fibres or striation in *T. cruzi*; while Das Gupta and others (1954) observed myoneme fibrils in *L. donovani*.

#### MATERIAL AND METHODS

The 'leptomonas' forms of the flagellate *H. muscarum* (family Trypanosomatidae), obtained from the gut of house-fly, were examined in physiological solution (Baker, 1944) by phase-contrast microscopy. Janus green B and neutral red were used for supervital staining.

For histochemical study, wet smears were employed. In some cases gelatine or paraffin sections of the gut containing the specimens were employed. Details of the histochemical tests used are given in the Appendix (pp. 418-19).

Specimens were also fixed in Lewitsky, Champy, or Helly, and stained with Heidenhain's haematoxylin or with acid fuchsin (Cain, 1948b).

#### RESULTS

The results of the cytochemical tests are set out in full in the Appendix (pp. 418-19).

The cytoplasm contains a parabasal body, mitochondria, and so-called 'volutin' granules.

*Parabasal body.* This appears as a duplex structure consisting of a dark externum and a light internum, in living specimens examined under the phase-contrast microscope. The externum of the parabasal body is darkened by 2% osmium tetroxide solution (fig. 1, A) and by Sudan black B (fig. 1, B), mercuric-bromophenol blue colours it (fig. 1, C). By all these techniques it appears crescentic. It appears blue in pyronin / methyl green preparations (fig. 1, D), and stains with iron haematoxylin in material fixed in Lewitsky, Champy, or Helly (fig. 1, E-G). It reacts positively to Feulgen's test.

The internum is not coloured by Sudan black even after the 'unmasking' techniques' of Ciaccio (1926) or Bradbury and Clayton (1958); in fact it is negative to all the histochemical tests that were tried.

The evidence suggests that the externum of the duplex parabasal body consists of neutral lipid (probably triglyceride), lipoprotein, and DNA. Because of the presence of DNA, the parabasal body has been regarded as a secondary nucleus or kinetonucleus by Fantham (1912) and Grasse (1926) in *Herpetomonas*, and by Sen Gupta and others (1953) in *L. donovani*. The duplex nature of 'kinetonucleus' or parabasal body in *Herpetomonas* was described by Grasse (1926) also. This parabasal body, containin

DNA, cannot be homologized with the 'Golgi apparatus' as Duboscq and Grassé (1933) suggested. The composition of the internum could not be determined.

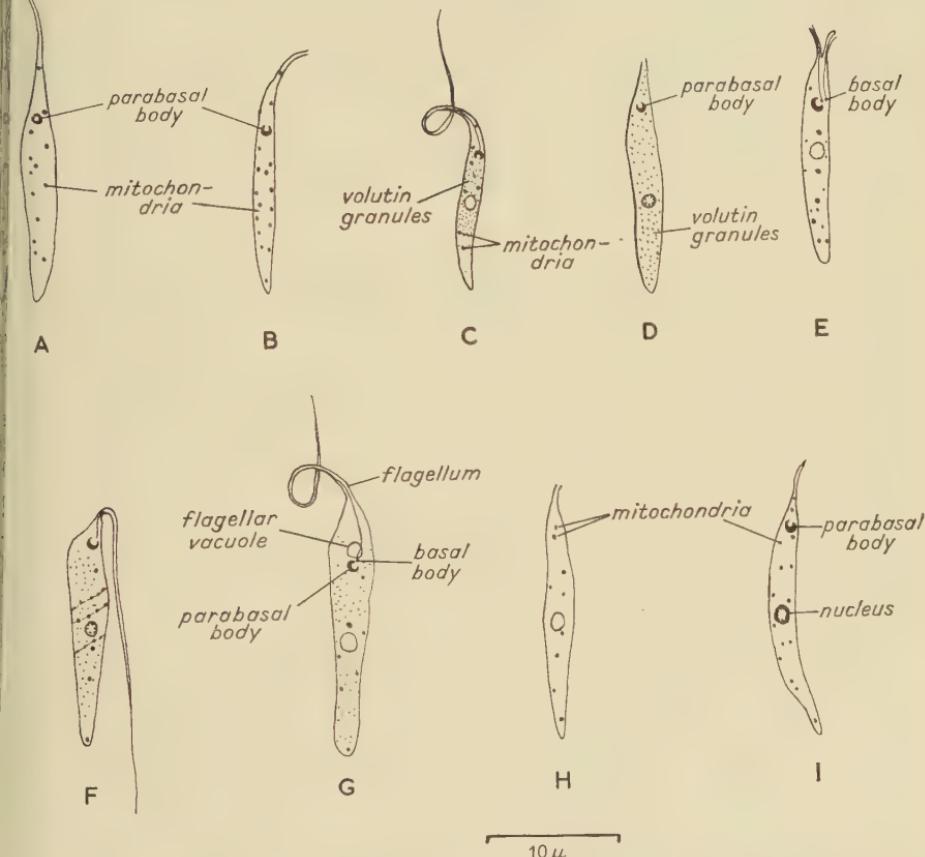


FIG. 1. Camera lucida drawings of *H. muscarum*. A, fresh specimen treated with 2% osmium tetroxide. B, formaldehyde-calcium, postchromed, Sudan black. C, weak Bouin, pyridine extraction, mercuric-bromophenol blue. D, Zenker, pyronin G / methyl green. E and F, Lewitsky, iron haematoxylin. G, Helly, iron haematoxylin. H, Helly, Cain's acid fuchsin. I, formaldehyde-calcium, mercuric bromophenol blue.

The basal body (blepharoplast) of the flagellum lies close to the parabasal body, and a flagellar vacuole lies on one side of the flagellum near its base (fig. 1, G). The basal body is stained by mercuric-bromophenol blue and also by iron haematoxylin. The 'cone of fibrillae', which connects the parabasal body with the blepharoplast according to Wilson (1925) and Wenyon (1926), could not be observed.

*Mitochondria.* These are in the form of granules scattered irregularly in the cytoplasm, or sometimes they are arranged along the inner surface of the

periplast (fig. 1, F). They are larger than the volutin granules. They are stainable by mitochondrial techniques (Janus green B; acid fuchsine (fig. 1, H). They are also darkly stained in iron haematoxylin preparations (fig. 1, E-G).

Khajuria (1950) denied the presence of mitochondria in this species. Studies by electron microscopy (Anderson and others, 1956; Chang, 1956) have also shown that mitochondria possessing the typical internal structure cannot be identified with certainty in *T. equiperdum* and *L. donovani*.

Guha and others (1956) and Sen Gupta and others (1953), however, have identified mitochondria in *L. donovani* by cytochemical methods. The former authors have employed specific enzymological tests, such as oxidation of the Nadi reagent and reduction of tetrazolium; and their conclusion seems to be beyond doubt. Causey (1927) described the presence of mitochondria in *L. brasiliensis*.

It appears probable that in the family Trypanosomidae, mitochondria do not possess the typical ultra-structure that can be identified with certainty by electron microscopy. Future studies by electron microscopy will probably establish with certainty the presence of mitochondria in these forms.

*'Volutin' granules.* They are more numerous than the mitochondria and are evenly distributed in the cytoplasm. They are specifically stained red in pyronin / methyl green preparations (fig. 1, D). They are not coloured by mercuric-bromophenol blue in material fixed in formaldehyde or formaldehyde-calcium (fig. 1, I), but are stained darkly after extraction with lipid solvents (fig. 1, C). Fixation of protein by formaldehyde is probably incomplete (compare Baker, 1958).

The volutin granules are weakly stained in iron haematoxylin preparations (fig. 1, F-G). They are not stained supervitally with Janus green B or neutral red.

Positive reactions for RNA and protein suggest the presence of ribonucleoprotein in them. Similar volutin granules composed of ribonucleoprotein have been described in other genera of Trypanosomidae by Berge (1942, 1946), Anderson and others (1956), and Sen Gupta and others (1953).

*Nucleus.* The nucleus is Feulgen-positive. It shows small dots arranged along the periphery of a ring when it is stained by methyl green in pyronin / methyl green preparations (fig. 1, D).

*Cytoplasm.* The diffuse staining of the cytoplasm by pyronin and mercuric-bromophenol blue suggests the presence of RNA and protein. Glycogen is uniformly dispersed in the cytoplasm.

If supervital preparations are kept for a long time in neutral red, small red vacuoles begin to appear in the cytoplasm. These vacuoles appear to be new formations. Baker (1958) has described the appearance of similar vacuoles on prolonged treatment with neutral red.

*Periplast.* The periplast or pellicle covers the cytoplasm on all sides and shows nearly parallel longitudinal striations (fig. 1, F). Similar striations have been described by Prowazek (1904), Chatton and Leger (1911), Kofoid and McCullock (1916), Kleinschmidt and Kinder (1950), Kraneveld and others (1951), Meyer and Porter (1954), and Das Gupta and others (1954).

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APPENDIX  
*The histochemical reactions of Herpetomonas muscarum*

Technique	Fixation	Reference	Parabasal body	Mitochondria	'Volutin' granules	Nucleus	Cytoplasm
SB in 70% ethanol	FCa and F Ca+PC	Baker, 1956	++c ++c ++c	+++ +++ ++c	-	-	-
SB in 70% ethanol at 60° C	"	Chiffelle and Putt, 1951	-	-	-	-	-
SB in propylene glycol	"	Krishna, 1950; Pearse, 1954	-	-	-	-	-
SB* cold acetone, ether or ethanol	Fresh or F Ca	Cain, 1947, 1948a	-	-	-	-	-
NB	FCa and F Ca+PC	"	-	-	-	-	-
NB* cold acetone	Fresh or F Ca	"	-	-	-	-	-
Sudan III and IV in 70% ethanol / acetone	FCa and F Ca+PC	Kay and Whitehead, 1941	-	-	-	-	-
Fettrot in 70% ethanol	"	Pearse, 1954	-	-	-	-	-
AH	FCa+PC	Baker, 1946	-	-	-	-	-
AH* PE	WB and PE	"	-	-	-	-	-
Fischler's reaction	F, F Ca	Pearse, 1954	-	-	-	-	-
Feyrter's enclosure	"	Schultz, 1924, 1925; Pearse, 1954; Gomori, 1952; Romieu, 1927	blue	blue	blue	blue	-
Cholesterol reactions	"	Pearse, 1951; Lillie, 1952	-	-	-	-	-
Performic acid / Schiff	F Ca	Nath, 1957	+	++	++	++	-
2% osmium tetroxide	Fresh	Bradbury, 1956b	+	++	++	++	-
Ciaccio's technique	F Ca and phenol	Bradbury and Clayton, 1958	++c	++c	++c	++c	-
Flemming unmasking	Flemming's+PC						-

2AS	B, C	Hotchkiss, 1948; Pearse, 1954	—
2AS*	acetylation	McManus and Cason, 1950	—
2AS* or N KOH	"	"	—
Best's carmine	"	Pearse, 1954	—
2AS or Best's carmine* salivary amylase	"	"	—
MHBB	F, F Ca	Mazia and others, 1953	—
MHBB* cold acetone, ether or ethanol	B, C, WB+PE	—	—
Feulgen	C	Pearse, 1954	red
Feulgen* trichloroacetic acid	Z	Schneider, 1945	blue
PP MG	"	Jordan and Baker, 1955	blue
PP MG* salivary ribonuclease	"	Bradbury, 1956a	—
PP MG* trichloroacetic acid	"	—	—

**KEY:** All acid haematein; B Bouin; C Carnoy, c = 'crescent'; F = formaldehyde; F Ca = formaldehyde-calcium; PE = pyridine extraction; P AS periodic acid / Schiff; PC with post-chromning; P/MG = pyronin G / methyl green; MBB = mercuric bromophenol blue; NB Nile blue; r ring; SB Sudan black B; WB and PE = weak Bouin followed by pyridine extraction; Z Zenker; + weak reaction; + + weak reaction; + + + strong reaction; — = negative; \* after treatment with.



# A New Method for Staining Connective Tissue Fibres, with a Note on Liang's Method for Nerve-fibres

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With two plates (figs. 1 and 2)

## SUMMARY

A new method of staining connective tissue fibres is described. The stain used is alcohol-soluble aniline blue (spirit blue), after oxidation with potassium permanganate. The method is rapid and easy in application. Liang's method for staining nerve-fibres is discussed briefly and its use in demonstrating the excretory system of *Fasciola* described.

## INTRODUCTION

CONNECTIVE tissue fibres, particularly the finer reticular fibres, are often demonstrated by silver impregnation techniques. In the majority of these methods the sections are first oxidized with potassium permanganate and decolorized by a rinse in sulphurous acid before metallic impregnation. In the method outlined here, sections are similarly treated with permanganate and sulphurous acid, but this is followed by a brief period in alcohol-soluble aniline blue, a stain rarely used in histological techniques. While not the equal of successful silver preparations, the method has proved simple and rapid in use, and demonstrates the distribution of connective tissue fibres in a variety of animals.

Fig. 1 illustrates the sub-epidermal region of A, a nemertine; B, *Lumbricus*; C, D, and E, *Peripatus*; and F, an ammocoete larva. The distribution of fibres in the sub-epidermal region of a nemertine (*Amphiporus*) has been described in detail by Cowey (1952) and it would appear that a somewhat similar system of fibres occurs in the earthworm. After treatment with the aniline-blue method outlined here, a system of fine reticular fibres can be demonstrated, particularly in the region of the circular muscle-layer (fig. 1, B). Sections of *Peripatus* and of ammocoete larvae treated in this way proved most interesting. Fig. 1, C shows a section of *Peripatus* treated with Mallory's triple stain. Beneath the epidermis of the integument is a layer of connective tissue, 15  $\mu$  thick, which colours blue and has a more or less homogenous appearance. After treatment with permanganate and spirit blue, the bulk of this layer remains colourless, or is coloured green if light green is used as a counterstain; but traversing the layer can now be seen numerous 'struts' of fine fibres coloured a deep blue (fig. 1, D). Beneath the epidermis these fibres are continuous with those of the basement membrane, while internally they are continuous with a system of fine anastomosing fibres surrounding the individual

muscle-fibres (fig. 1, E). This connective tissue 'skeleton' of *Peripatus* is of considerable interest in the light of Manton's (1958) discussion of habits and evolution of body design in the arthropods. A somewhat similar system of fibres can be demonstrated in the sub-epidermal connective tissue of ammocoete larvae (fig. 1, F). Figs. 2, A, B, C, illustrate preparations of various vertebrate tissues. Fig. 2, A shows the fibres in the submucosa of the frog's stomach; B, those of the muscularis externa of the small intestine of the cat; and C, those of the diaphragm of the cat.

The advantages of the permanganate / spirit blue method are its simplicity and its application to tissues fixed in a variety of fixatives. It is interesting to note that the method also stains neurosecretory material with results somewhat similar to those obtained with the paraldehyde/fuchsin technique (Gall 1953). This stain was used earlier by Gomori (1950) for elastic fibres, which are also demonstrated by the present method (fig. 2, A).

## METHOD

### Solutions

#### Oxidizing solution

Potassium permanganate . . . . .	0.5 g
Conc. sulphuric acid . . . . .	0.5 ml
Water . . . . .	100 ml

This solution remains effective for approximately 2 h. The concentration of potassium permanganate is not critical.

#### Decolorizing solution

Potassium bisulphite . . . . .	1.0 g
Normal hydrochloric acid . . . . .	1.0 ml
Water . . . . .	99 ml

#### Stain solution

Alcohol-soluble aniline blue ('michrome' brand) . . . . .	0.5-1.0 g
Aniline oil . . . . .	1.0 ml
70% alcohol . . . . .	99 ml

The concentration of the stain can be varied to suit various tissues.

FIG. 1 (plate). A, sub-epidermal region of a hoplonemertine. Fixed in Bouin's fluid and treated with potassium permanganate spirit blue.

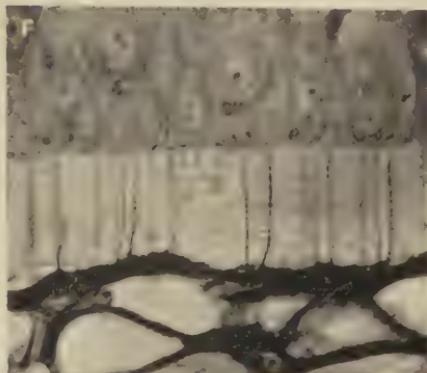
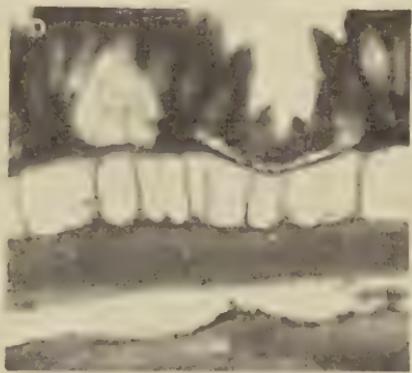
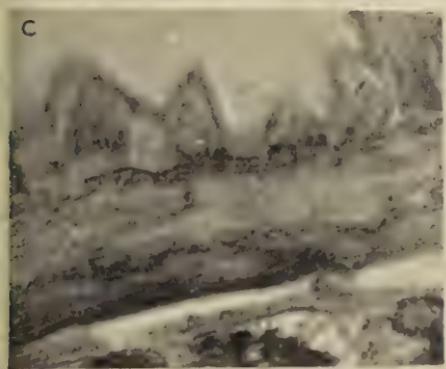
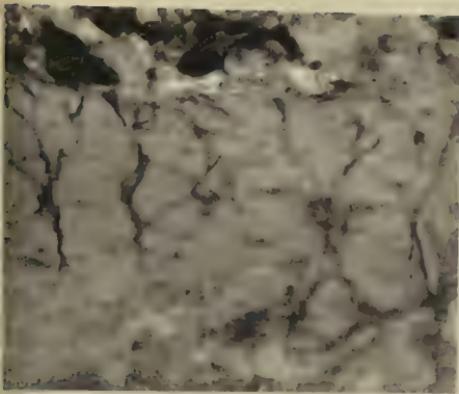
B, T.S. *Lumbricus*, showing connective tissue fibres traversing the layer of circular muscle. Fixed and stained as in A.

C, T.S. *Peripatus*, showing the integument, connective tissue layer, and circular muscle. Fixed in modified Bouin-Dubosq (Atkins, 1937) followed by Mallory's triple stain.

D, as C but treated with potassium permanganate / spirit blue in place of Mallory's triple stain. Note the 'strut'-like system of fibres traversing the layer of connective tissue.

E, staining as D but showing the connexion between the 'strut'-like fibres and the system of fibres investing a circular muscle fibre.

F, sub-epidermal region of an ammocoete larva. Fixed in Bouin's fluid and stained as in D.

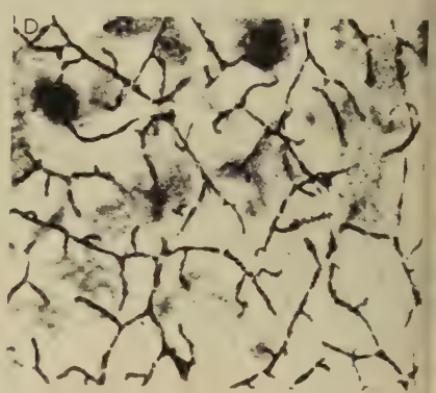
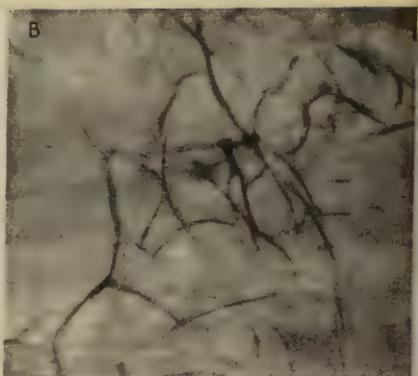
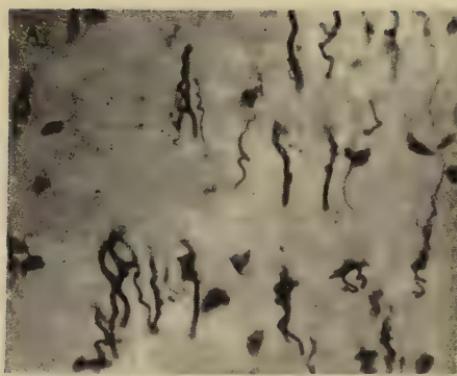


$10\ \mu$   
A & B

$20\ \mu$   
C & D

$10\ \mu$   
E & F

FIG. 1  
G. OWEN



$10\ \mu$   
A B & C

$200\ \mu$   
D

$400\ \mu$   
E

FIG. 2  
G. OWEN

cedure

- 1. Bring sections to water in the usual way.
- 2. Oxidize in the potassium permanganate solution for 1 min.
- 3. Rinse in distilled water (30 sec).
- 4. Decolorize in sulphite solution (approximately 30 sec).
- 5. Rinse well in distilled water (1-2 min).
- 6. Stain in aniline blue (2 min).
- 7. Rinse in water to remove excess stain.
- 8. Transfer directly to absolute alcohol and differentiate (with some tissues this may take place rapidly).
- 9. Clear in xylene and mount in balsam.

The tissues will have a general background colour of light blue while connective tissue fibres will be coloured intense blue. A solution of light green (0.5-1.0% in 1% aqueous acetic acid) may be used as a counterstain approximately 5 sec) after stage 7. If this is used, the time of differentiation in absolute alcohol is reduced.

#### *Ang's method of using the Schiff reaction for nerve staining*

Liang (1947) reported a new method for staining nerve-fibres and endings with the Schiff reagent. This has been used in this Department and the following comments are of interest. It should be pointed out that the method has not proved successful in demonstrating motor-nerve end-plates. Nevertheless, it has provided a simple and rapid method of demonstrating the general distribution of nerve-fibres in a variety of animals and tissues. In particular, it has been used successfully with bivalve and gastropod molluscs, animals in which it is notoriously difficult to demonstrate the peripheral nervous system with the more familiar methylene blue and metallic impregnation techniques. Fig. 2, is a portion of a whole mount of the siphon of *Buccinum* (Gastropoda) prepared by Mr. P. S. Maitland, of this Department, by using the Schiff reagent. It demonstrates most clearly the rich innervation of this organ. For the preparation of whole mounts, or imbedding in wax and subsequent sectioning, Liang recommended rapid dehydration in the alcohols. It has been found, however, that the purple colour of the fine nerve-fibres is rapidly removed in both alcohol and cellosolve, and it is not possible to obtain successful preparations in this way. Instead, tissues should be transferred directly to several changes of absolute acetone and then to xylene. Mount in balsam.

FIG. 2 (plate). A, submucosa of frog's stomach. Fixed in 1% formic acid and treated with potassium permanganate / spirit blue.

B, muscularis externa of the small intestine of the cat. Fixed in Zenker's fluid; staining as in A.

C, diaphragm of cat. Fixed in 1% formic acid; staining as in A.

D, excretory system of *Fasciola hepatica* (whole mount). Fixed in 1% formic acid; staining as in Liang's method but dehydrated in absolute acetone; cleared in xylene and mounted in balsam.

E, siphon of *Buccinum undatum* showing nerve supply (whole mount). Treatment as in D.

or embed in wax. The whole mount of the siphon of *Buccinum* (fig. 2, E) was prepared in this way.

One further point is of interest. Fresh specimens of the liver fluke, *Fasciola*, were subjected to Liang's method but failed to show any trace of the nervous system. It was successful, however, in demonstrating in detail the excretory system, a portion of which is shown in fig. 2, D. The granular contents of the peripheral excretory ducts are coloured an intense red.

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# Sections of Fresh Mammalian Nerve-trunks for Quantitative Studies: A Rapid Freezing Technique

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With three plates (figs. 2 to 4)

## SUMMARY

Attempts to assess the effects of shrinkage and distortion inherent in the conventional histological procedures used during quantitative analysis of nerve structure have hitherto proved unsatisfactory. The evolution of a technique in which such effects are minimized is therefore desirable.

A segment of fresh nerve, implanted in a mass of supporting tissue (fresh liver), is frozen by a solid  $\text{CO}_2$ /acetone mixture, and sectioning is subsequently performed in a cryostat. The sections are flattened on pre-cooled slides, irrigated with saline, and photographed.

Regular, stable serial sections, 3 to 5  $\mu$  in thickness, may be produced. Such sections show typical internodal annuli of compact myelin, shearing defects of Schmidt-Ganterman type, and paranodal crenations of the myelin sheath. Occasional defects of separation include rupture of isolated fibres, herniation of myelin, and the appearance of myelin spheres.

When studies involving precise measurement are contemplated, the use of orthodox transmitted illumination, dark-ground illumination, and phase contrast is precluded by diffraction effects and the occurrence of reverse-contrast haloes at the tissue interfaces. Polarizing microscopy has proved satisfactory.

It has been shown that estimates of the relative size of the axon and myelin sheath, obtained from sections prepared in this manner, do not differ significantly from the relative sizes measured in fibres freshly teased in physiological saline (Wendell-Smith and Williams, 1959).

## INTRODUCTION

THE earliest recognition of the essential fibrous nature of peripheral nerve (Monro, 1779; Fontana, 1781) was followed by sporadic attempts to determine the relative sizes of different component parts of various fibre populations (Bidder and Volkmann, 1842; Schwalbe, 1882; Gaskell, 1886; Schiller, 1889).

The researches of Sherrington (1894) and Eccles and Sherrington (1930) on the innervation of skeletal muscle, and the efforts of Erlanger and Gasser (1937) to correlate structural features with the findings of electrophysiology, stimulated interest in more systematic quantitative analyses of nerve structure.

Determinations of the frequency distribution of diameters have been used by many investigators as a basis for the classification of nerve bundles. In such studies, the external diameter (i.e. the total diameter including the compact myelin sheath) of the individual fibres is estimated. The principal findings of such investigations on muscle-nerves have been reviewed by Hinsey (1934),

Rexed (1944), Fernand and Young (1951), and Tiegs (1953); and on cutaneous nerves by Davenport and others (1934), Sanders and Young (1944), Lavar and others (1949, 1951), and Quilliam (1955, 1956).

Factors which may operate in the determination and control of fibre diameter during growth and regeneration have been considered by Aitken and others (1947), Sanders and Young (1944, 1945, 1946), and Fernand and Young (1951).

Diameter determinations have been of importance during studies of shear birefringence (Schmitt and Bear, 1939; Taylor, 1942) and also in the attempt correlation of structure with physiological data (Erlanger and Gasser, 1933; Hursh, 1939; Gasser, 1938, 1941; Grundfest, 1939, 1940; Gasser and Grundfest, 1939). Recent theoretical considerations of the structural basis of saltatory conduction (Rushton, 1951) have emphasized the need for accurate information concerning the relationships that may exist between axon diameter, thickness of myelin sheath, internodal distance, and the various dimensions of the nodal apparatus.

Such investigations are dependent upon methods of linear measurement applied to various types of histological preparation. Techniques commonly employed include treatment with osmium tetroxide, the Alzheimer-Manz-Haggqvist technique, and modifications of the Weigert-Pal technique. Quantitative assessments of the effects of shrinkage and distortion inherent in the methods (Sherrington, 1894; Duncan, 1934; Arnell, 1936; Taylor, 1942; Rexed, 1944; Sanders, 1948) provide conflicting results. Such effects are minimized if methods of sectioning involving rapid freezing of the fresh nerve-trunk are employed. Orthodox frozen sectioning methods (Arnell, 1936; Rexed, 1944) have in general proved unsatisfactory. Undesirable features of such sections (Rexed, 1944) include irregularity, excessive thickness ( $> 10 \mu$ ) and a tendency to disrupt during subsequent processing. In addition there is instability of fibre contour, the difficulty of producing complete sections of a nerve-trunk, and the impossibility of serial sectioning.

The technique to be described allows the production of regular, stable serial sections, 3 to 5  $\mu$  thick, of fresh nerve-trunks.

#### MATERIAL AND METHOD

The sciatic nerve, the sural nerve, and the nerve to the medial head of the gastrocnemius (N.G.M.) of the rabbit have been used throughout the present investigation.

The technique consists of:

- (1) preparation of blocks of supporting tissue (e.g. fresh liver);
- (2) implantation of the nerve segment in the supporting tissue;
- (3) freezing the block;
- (4) sectioning in a cryostat at  $-18^\circ \text{C}$ ;
- (5) section mounting, saline irrigation, and photomicrography.

*Preparation of supporting tissue blocks.* The presence of a mass of supporti

tissue surrounding the segment of nerve facilitates handling of individual sections, allows greater control of the freezing process, and prevents disintegration of the margins of the sections. Initially, segments of spinal cord were used as an embedding medium but this proved less satisfactory than blocks of fresh liver. Under nembutal anaesthesia the liver is removed and a block approximately  $2 \times 1 \times 1$  cm prepared with a sharp scalpel. To facilitate cutting, the liver should be supported on a plane glass surface (fig. 1, A). The

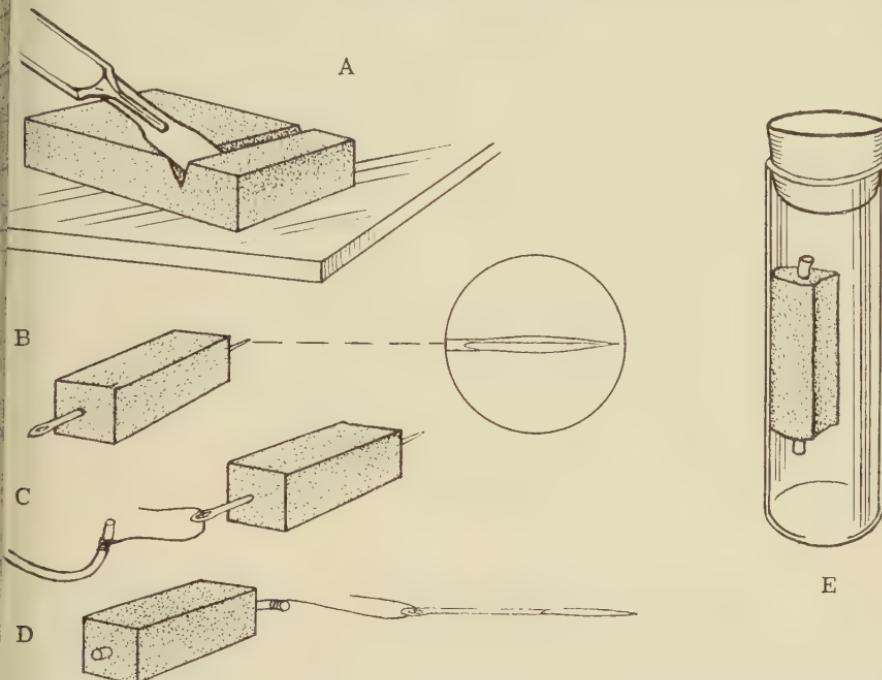


FIG. 1. Implantation of the nerve in supporting tissue. A, cutting the block of liver. B, preparation of the reception channel. C, D, implantation of the segment of nerve. E, block adhering to the side wall of a glass tube before freezing.

piece of liver selected should be free from large radicles of the hepatic vascular and duct systems, as these may cause difficulty during implantation and subsequent sectioning. A channel to receive the nerve is prepared by passing a straight cutting needle 10 to 12 times through the long axis of the block (fig. 1, B). The diameter of the needle should be slightly in excess of that of the nerve to be embedded.

*Implantation of the nerve.* The nerve is gently freed and a fine silk ligature attached to its proximal extremity. Stripping of the perineurium is avoided as this results in damage to and subsequent detachment of superficial fibres. The segment of nerve including the ligature is excised. The portion of nerve included proximal to the ligature should not exceed 2 mm in length. The straight needle is used to thread the ligature through the reception channel, and gentle traction on the ligature causes the nerve to be drawn into position

within the block (fig. 1, c, d). Gentleness is essential during this manoeuvre. Common causes of difficulty include inadequate preparation of the reception channel and the use of a coarse ligature resulting in a bulky knot.

**Freezing.** The block is placed against the side wall of a glass tube of suitable dimensions (fig. 1, e). The tube is corked securely, immersed in a mixture of acetone and solid carbon dioxide contained in a vacuum flask, left for 20 min and then transferred to the chamber of a cryostat.

**Sectioning.** The cryostat used was similar to that described by Coons, Leduc, and Kaplan (1951). It provides a large chamber controlled at  $-18^{\circ}\text{C}$  in which microtomy, section flattening, and attachment to slide are performed. Standard forms of the Cambridge rocking microtome and the Cambridge rotary-rocking microtome, each provided with a glass section-flattening device (Coons and others, 1951), proved satisfactory. Sectioning is started at  $15\text{ }\mu$  and continued until the first few mm of the block have been removed. Cutting is then continued at the desired thickness (consecutive sections 3 to  $5\text{ }\mu$  may be mounted with comparative ease). Individual sections are placed on pre-cooled slides which have been stored in the cryostat after preliminary flattening effected by a few strokes of a fine camel-hair brush. The slide is removed from the cryostat. Section flattening and attachment to the slide are completed by a process of thawing induced by placing the ungloved finger on the reverse side of the slide. Excess moisture is allowed to evaporate by exposure to the air at room temperature for a period of 1-2 min. The section is irrigated with physiological saline and photomicrographs are taken.

#### OBSERVATIONS AND DISCUSSION

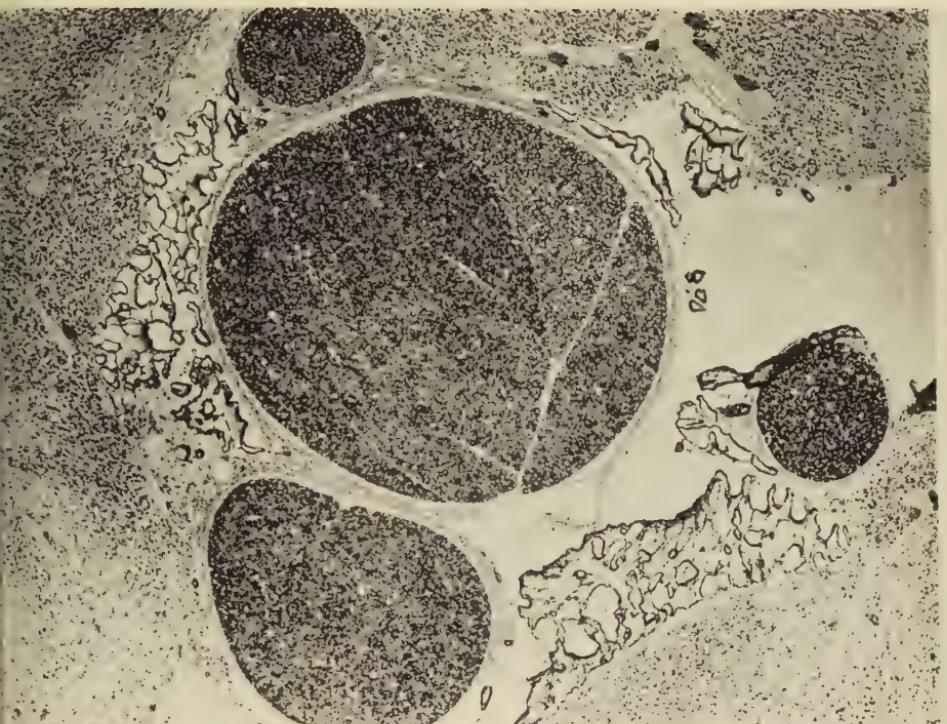
Various forms of microscopy have been employed to assist interpretation of the appearances of unstained sections prepared in the manner described.

**Transmitted light.** The appearance of a low-power view with Köhler illumination of a  $5\text{ }\mu$  transverse section of the sciatic nerve of the rabbit is shown in fig. 2, A. The supporting tissue, the continuity of the perineurium, and the regularity and integrity of the individual fascicles may be seen. Fig. 2, B, illustrates a higher power view of the nerve to the medial head of the gastrocnemius (N.G.M.). The only tissue-component to be visualized with ease is the sheath of compact myelin. The region of the axon is featureless and transparent, and the endoneurium, neurolemma, Schwann cell cytoplasm and nuclei are indistinguishable. A section through a typical internode shows a regular annulus of myelin (fig. 3, B). The external and internal surfaces of the myelin are not sharp, but present zones of diminished intensity, due largely to intense diffraction occurring at the tissue interfaces. The form of

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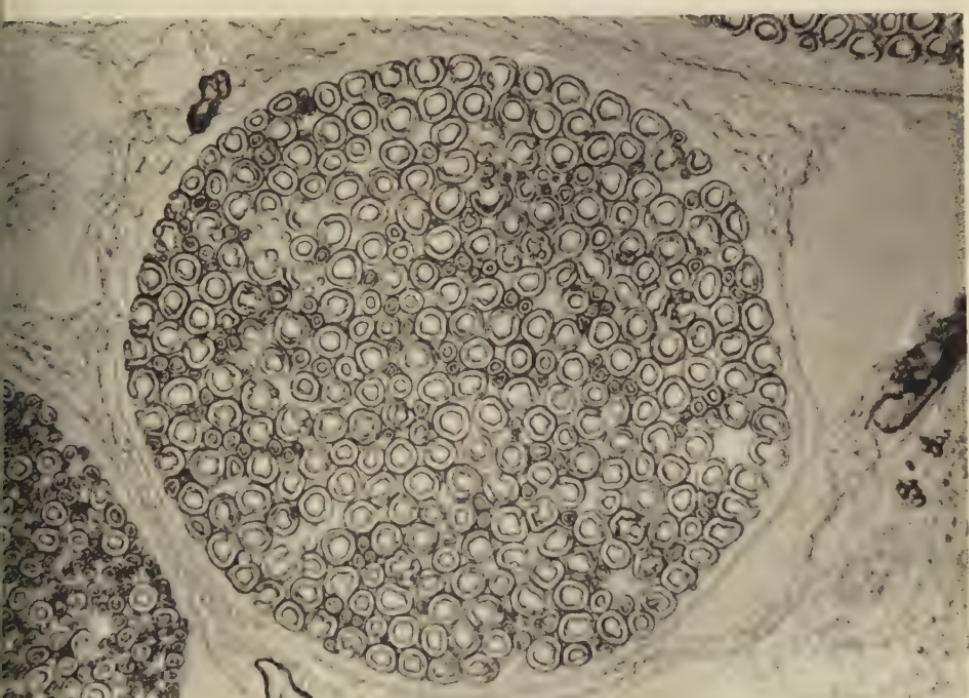
FIG. 2 (plate). A, sciatic nerve of the rabbit,  $5\mu$  transverse section; Köhler illumination. The supporting tissue, the perineurium, and the regularity and integrity of the individual fascicles may be seen.

B, *nervus gastrocnemius medialis* of the rabbit,  $5\mu$  transverse section; Köhler illumination.



A

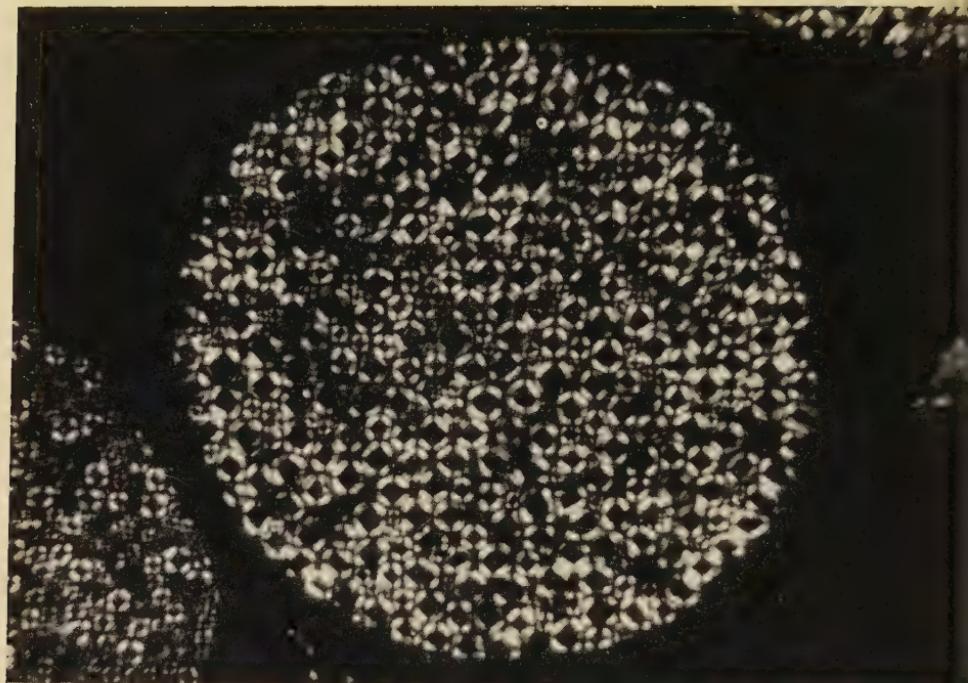
$200\text{ }\mu$



B

$30\text{ }\mu$

FIG. 2  
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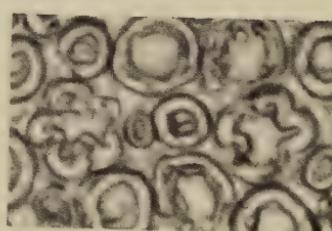


A

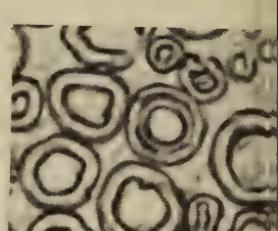
$30\mu$



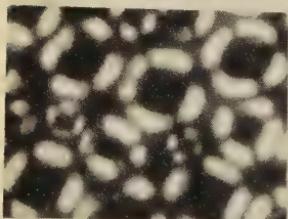
B



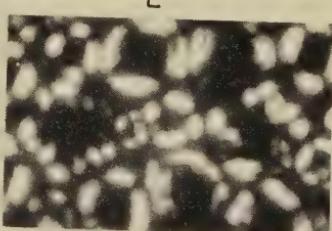
E



H



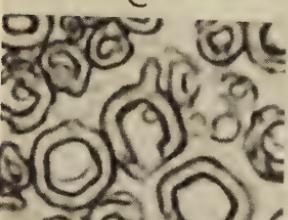
C



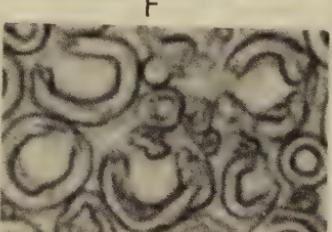
F



I



D



G

$10\mu$

FIG. 3

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e diffraction gradients that occur at interfaces of this nature, and the sultant difficulties in the performance of precise measurement, have been scussed by Ross (1957). Where the plane of section has passed through a earing defect of Schmidt-Lantermann type (Robertson, 1958), the myelin eath appears as two well-defined, concentric laminae (fig. 3, H, I).

The paranodal crenations of the myelin sheath that have been described in xed preparations by various workers (Ranvier, 1878; Key and Retzius, 1876; ageotte, 1922; Hess and Young, 1952; Quilliam, 1956) may be observed (fig. 3, E, F).

Occasional fibres may show fragmentation of the sheath, herniation of the yelin, or the appearance of isolated myelin spheres (fig. 3, D, G, J). In well- epared sections such appearances are infrequent and a high incidence of ch forms may be taken as an index of inadequate preparation.

*Dark-ground illumination* (fig. 4, A). With this form of illumination the tense scatter of light occurring at the external and internal surfaces of the compact myelin is emphasized.

*Phase contrast* (fig. 4, B). All the features described for ordinary transmitted lumination may be appreciated. The axonal area appears homogeneous, and here is no evidence of a periaxonic layer of Schwann-cell cytoplasm of the relatively large proportions described by Esmond and Smith (1958).

The precise external and internal limits of the zone of compact myelin are bscured by the occurrence of reverse-contrast haloes, which accompany hase contrast techniques (Barer, 1956).

*Polarizing microscopy*. The general optical properties and characteristic rms of anisotropy exhibited by vertebrate myelin sheaths under various xperimental conditions have been discussed by Schmidt and Bear (1939). The internodal myelin sheath may be regarded as a system of positive uniaxial rodlets, the long axis and optic axis of each rodlet being coincident and aving a radial disposition. The general appearance of a fresh transverse ection of the N.G.M. of the rabbit, photographed between crossed polaroids without a compensator, is seen in fig. 3, A. The annuli of compact myelin are rightly illuminated but exhibit four points of extinction giving rise to the typical 'Maltese-cross' appearance (fig. 3, C). The axoplasm and endoneurium re not brightly illuminated. This observation is in keeping with the structural

FIG. 3 (plate). *Nervus gastrocnemius medialis* of the rabbit,  $5\mu$  transverse sections. A, polarized light.

B, Köhler illumination. A group of internodes. Note the zones of diminished intensity at the inner and outer surfaces of the myelin sheath.

C, polarized light. A group of internodes. The Maltese cross of polarization is evident.

D, Köhler illumination. Herniation of myelin.

E, Köhler illumination. Paranodal crenations of the myelin sheath.

F, polarized light. Paranodal crenations of the myelin sheath.

G, Köhler illumination. Ruptured fibres.

H, Köhler illumination. Shearing defect of Schmidt-Lantermann type.

I, polarized light. Shearing defect of Schmidt-Lantermann type.

J, Köhler illumination. The formation of myelin spheres.

The  $10\mu$  scale applies to B-J.

organization and form of anisotropy exhibited by these structures. The illuminated segments of myelin present well-defined external and internal boundaries which contrast sharply with the dark background and facilitate measurement. Estimates of external and internal fibre diameter are made from the centre of diametrically opposed luminous segments. The appearance of shearing defects of Schmidt-Lantermann type and also paranodal crenations when polarized light is used are seen in fig. 3, F, I.

The views of Young (1945) and Lubinska (1952, 1954, 1956 a, b) concerning the fluid nature of axoplasm and myelin might suggest that sections prepared in the manner described would be in an unstable state. Continued observation of individual sections shows, however, that fibre contour and size relations are unaltered for considerable periods after preparation, and only after immersion in saline for several hours does a slow but progressive deterioration occur. Possibly the method of preparation is accompanied by gelation of the axoplasm and consequent preservation of form (Lubinska, 1952, 1956 a, b). However, it must be admitted that views concerning the physical properties of axoplasm and myelin in the natural state are still highly speculative.

When considering the use of such sections in quantitative studies it is important to know what relation they bear to other types of preparation and to the natural state. It has been shown that the size relations present in frozen sections do not differ significantly from those present in fibres carefully teased in physiological saline. (Wendell-Smith and Williams, 1959.)

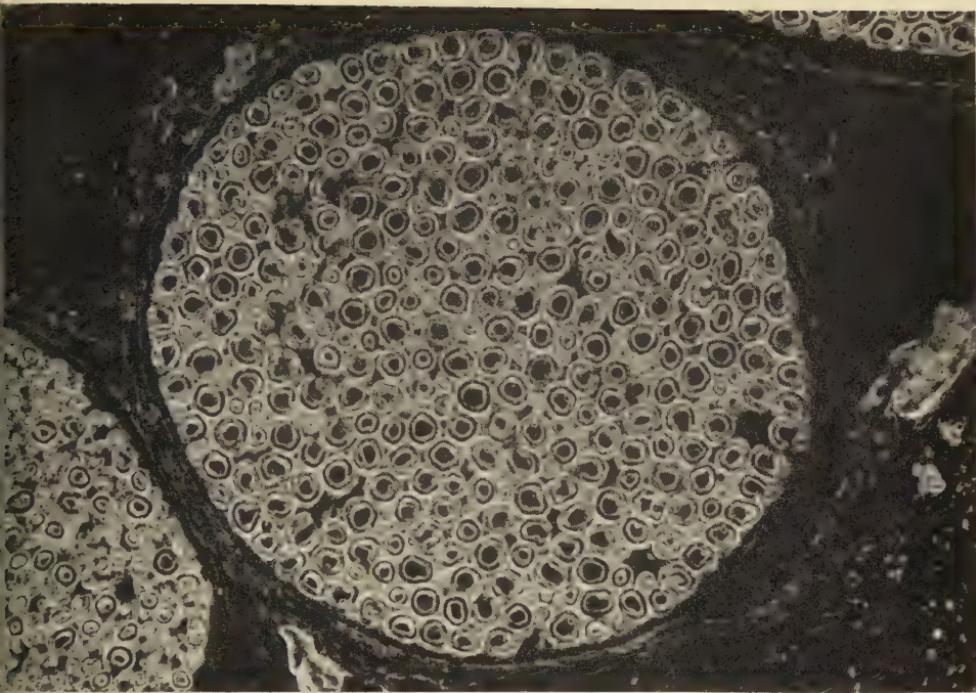
I am indebted to Professor R. Warwick for continued interest and criticism. The technical assistance and photography of Mr. A. N. Finch have been invaluable during the evolution of the technique. The line diagram was prepared by the Medical Illustrations Department, Guy's Hospital. This work has been supported by a grant from the Central Research Fund, University of London.

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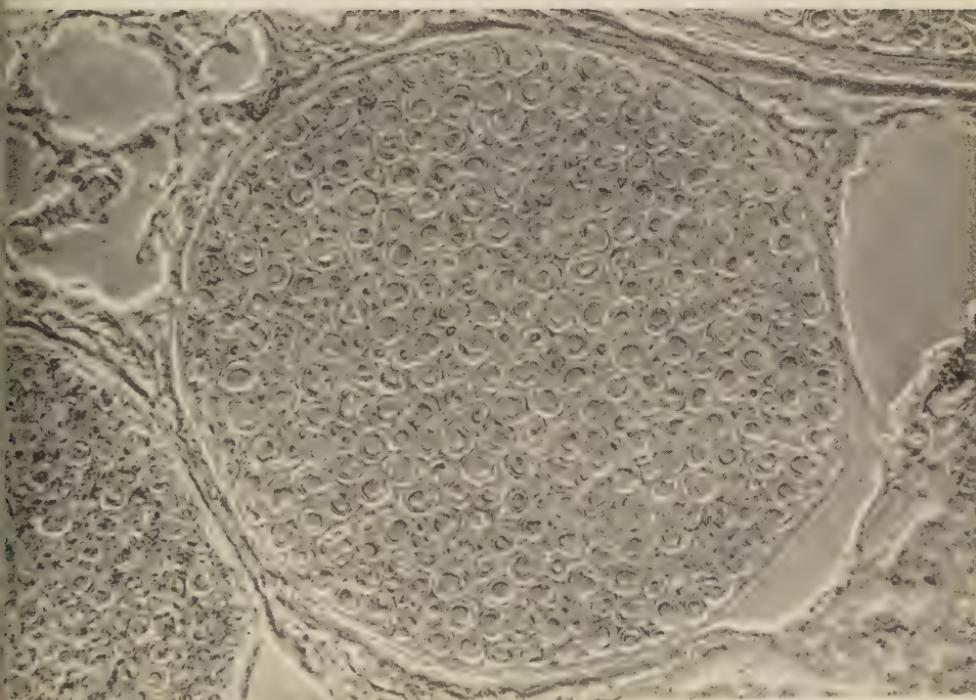
FIG. 4 (plate). *Nervus gastrocnemius medialis* of the rabbit,  $5\mu$  transverse section. A, dark ground. Intense scatter of light occurs at the internal and external surfaces of the compact myelin.

B, phase contrast. Note the occurrence of reversed-contrast haloes at the tissue interfaces



A

$30\mu$



B

$30\mu$

FIG. 4

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# The Association of a Slime Bacterium with the Inner Envelope of the Egg of *Dytiscus marginalis* (Coleoptera), and the less common Occurrence of a similar Bacterium on the Egg of *D. semisulcatus*

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With two plates (figs. 1 and 4)

## SUMMARY

The egg of *Dytiscus marginalis* L. is laid under water in the mesophyll of the leaf of various aquatic plants. The egg increases in size during development and this results in the splitting of the chorion, the outer envelope of the egg, usually into two halves. The inner envelope of the egg, the vitelline membrane, then forms the only complete shell of the egg in its later stages.

During the development of the embryo of *D. marginalis* the cysts of a slime bacterium (*myxobacteriale*) appear in great numbers on the vitelline membrane along the dorsal surface of the embryo and around each end of the egg. They are at first small and scattered, but by the time the larva hatches they are present in dense colonies. These bacterial cysts occur on eggs collected from water plants in ponds; but they develop just as readily on eggs obtained in the laboratory by placing the female *D. marginalis* in jars of tap-water with leaves that have never before been immersed in water.

Slime bacteria in the vegetative stage are carried in great numbers by the female *D. marginalis*, as has been shown by trailing the apex of the abdomen of a living beetle over an agar culture, when swarms of the characteristic rod-like cells have been obtained. Some of these bacteria will be transferred from the beetle to the egg during oviposition.

The bacterial cysts do not occur on unhealthy eggs and they rarely show normal development on eggs parasitized by the mymarid, *Caraphractus cinctus* Walker. This suggests that their development is dependent upon the healthy growth of the *Dytiscus* embryo.

In the egg of *D. semisulcatus* Müll. the chorion remains in close contact with the vitelline membrane throughout embryonic development and only occasionally shows small irregular cracks before hatching. On some eggs of this species a few scattered cysts of slime bacteria occur on the vitelline membrane. On occasional eggs they were more numerous, but they were never observed to be present in sufficient numbers to form the even covering which is so striking a feature of certain parts of the egg of *D. marginalis*.

## INTRODUCTION

FOR some years I have been breeding the mymarid, *Caraphractus cinctus* Walker, on the eggs of various water beetles of the genera *Agabus*, *Ilybius*, *Polynotes*, and *Dytiscus* (Jackson, 1958). I have thus had occasion to examine the eggs of all these species closely at all stages of their development and I have been surprised to find that, in eggs of *D. marginalis* in which the embryo

was well developed or from which the larva had hatched, minute greenish spheres were almost invariably to be found in great numbers in any microscopic preparation made of the egg envelopes (fig. 1, A, B). The minute spheres were restricted to certain parts of the inner membrane, the vitelline membrane, and they never occurred on the chorion. It was at first thought that these objects were algae, but they have now been identified by Dr. J. W. G. Lund and Miss V. G. Collins as cysts of one of the slime bacteria of the order Myxobacterales.

These bacterial cysts occurring on the *Dytiscus* egg do not appear to be comparable with the various micro-organisms, usually believed to be symbionts, which occur intracellularly or intercellularly in a variety of insects, and which are transmitted from generation to generation in various ways (Buchner, 1953). Thus, the bacteria studied by Gier (1936) in roaches, and termed by him bacteroids, are present in certain cells (mycetocytes) in the fat-body. These bacteroids also occur in the ovary, between the oocyte membrane and the follicle cells, and they later enter the egg cytoplasm. They retain their position against the vitelline membrane after the egg is laid, and, as the embryo develops, they move to the centre of the yolk. The slime bacterial cysts of the *Dytiscus* egg, on the contrary, have never been seen within the egg. They have only been found upon the vitelline membrane, where they appear during the later stages of embryonic development, and they persist on this membrane after the larva has hatched. In a variety of beetles which harbour yeasts or bacteria internally, the eggs become smeared with micro-organisms during oviposition, and the larvae on hatching eat a part of the contaminated shell and so become infected (Buchner, 1953). This could not happen with the *Dytiscus* larva since the larva is not in contact with the vitelline membrane before hatching, being completely enclosed in the embryonic cuticle (Jackson, 1957). When about to hatch the larva throws back its head and suddenly splits both the embryonic cuticle and the vitelline membrane, aided by the egg bursters on the frons (Balfour-Browne, 1913). It then bursts out of the egg into the water to seek its prey, and since it sucks the juices of its victim through a groove in each mandible (after injection of a digestive fluid), there can be no question of contamination with slime bacteria during feeding. As will be discussed later (p. 442) an external source of infection of the female *Dytiscus* indicated.

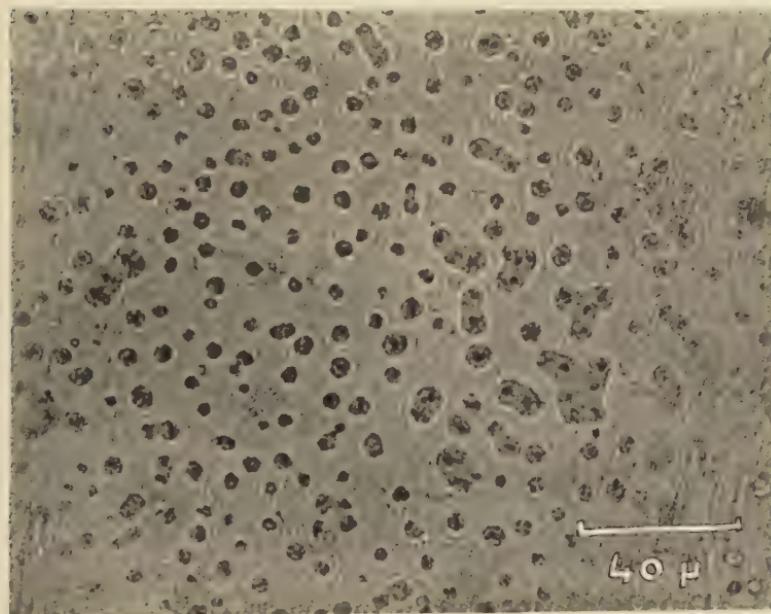
Since the myxobacterales are probably little known except to the specialist, it may be of interest to mention that these organisms, called myxobacteria or slime bacteria, consist in the vegetative stage of flexible rod-like cells which form a colony or swarm and multiply by transverse fission. According to

FIG. 1 (plate). A, photomicrograph of a portion of the vitelline membrane of a hatched egg of *D. marginalis* under low magnification, to show the distribution of the bacterial cysts. The Y-shaped base of the rent made by the larva in hatching shows at the top right-hand side. From a glycerine jelly mount.

B, photomicrograph of an area of the vitelline membrane from an egg of *D. marginalis* containing an embryo larva, to show the bacterial cysts. Mounted in polyvinyl lactophenol.



A



B

FIG. 1

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gey and others (1948) the cells may form a group which moves as a unit a crawling or creeping motion away from the centre of the colony, and the wing cells pave the substrate with a thin layer of slime on which they rest. In the fruiting-stage the rods ordinarily associate in clumps to form cysts, and in the cysts, in some forms, the rods may become ovoid or cylindrical, functioning as spores.

Most of the species have been obtained from soil and from the dung of various animals, sometimes also from lichens or decaying leaves, bark, &c., while some species of *Cytophaga* are marine. Most slime bacteria are saprophytic, some are believed to be facultative parasites on various bacteria. One species, *Chondrococcus columnaris* (Davis) Ordal and Rucker, is parasitic on an alga, while *Polyangium parasiticum* Geitler is parasitic on an alga (*Cladophora*) growing in fresh water and forms its cysts always under water (Geitler, 1925). As far as I am aware this is the first record of the occurrence of these bacterial cysts on the egg of a water-beetle, and, though Blunck (1914) has described the egg of *D. marginalis* in much detail, he makes no reference to them. I have found similar cysts present, usually in very small numbers, on some eggs of *semisulcatus*, but I have never found them on the eggs of water-beetles belonging to other genera, though the eggs of *Agabus* and *Ilybius* may be laid on the same aquatic plants, those of *Ilybius* being similarly buried in the leaf tissue. These bacterial cysts are more numerous on some eggs of *D. marginalis* than on others, but they show a fairly constant orientation on the vitelline membrane. Before describing their position it will be necessary to refer to the principal changes occurring in the envelopes of the egg of *D. marginalis* from oviposition to hatching. The condition of these envelopes in the egg of *semisulcatus* will be dealt with later.

#### *DYTISCUS MARGINALIS* L.

*Oviposition.* As is well known, the female *D. marginalis* inserts her egg by means of her powerful cutting ovipositor in the tissues of various plants growing under water. The process of laying has been described and figured by Blunck (1913) and his observations are included in Korschelt's monograph (1924). The long egg is so inserted in the mesophyll of the leaf that it is hidden from view, but, as the egg swells during its development, the epidermis of the shell becomes raised and the narrow slit through which the egg is laid widens appreciably. It is thus usually possible in the later stages of development to see the anterior end of the egg through this opening in the leaf (fig. 2). The egg is so inserted that the dorsal surface of the embryo is uppermost (on the outer side of an upright leaf), while the lower part of the head with the mandibles and other mouth-parts faces the inner part of the leaf. This is in accordance with Hallez's law (1886), the orientation of the egg corresponding to that of the mother, for, when laying, the beetle clasps the plant with its legs and projects the egg into the tissues of the leaf in line with its body, so that the head of the egg, dorsal side up, comes to lie under the slit made by the ovipositor.

*The egg envelopes.* In the newly laid egg of *D. marginalis* the chorion is closely applied to the vitelline membrane that the two membranes can only be distinguished in parts by transmitted light. The micropylar area shows distinctly in the chorion as a round white spot at the anterior end of the egg. When newly laid the egg is surrounded with gelatinous cement which stains pale green with methylene blue. The egg undergoes a great increase in size during development, and Blunck (1914, pp. 88-89) records that the egg swells

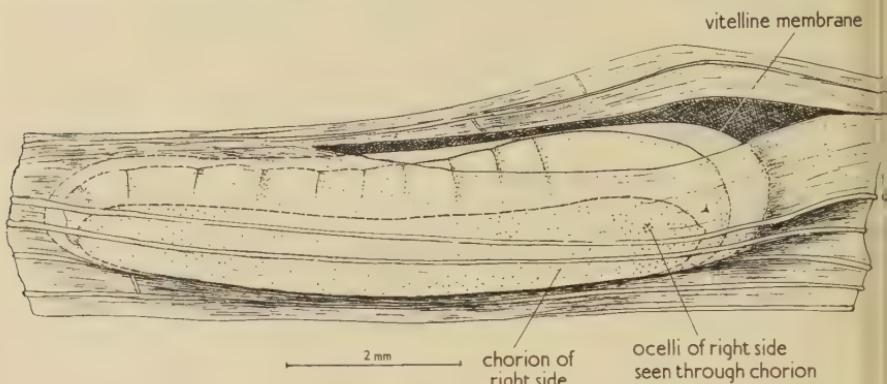


FIG. 2. Egg of *D. marginalis*, lateral view, with embryo, in leaf of *Juncus articulatus* L., to show the opening in the epidermis of the leaf originally made by the ovipositor of female *D. marginalis* and extended by swelling of the egg. The chorion of one side of the egg may be seen through the leaf, while the vitelline membrane projects beyond it and now forms the 'shell' of the egg. The egg-burster of one side shows as a V-shaped spine on the front of the head. From an egg fixed in picric acid in absolute alcohol.

from 1.2 to 2.25 mm in diameter, and increases in length by more than a millimetre. He states that in the course of embryonic development the chorion becomes entirely detached from the vitelline membrane and cracks. I have found that, as the egg increases in size, the chorion splits, usually into two equal halves, by a long slit down the dorsal and ventral surface of the embryo. The two halves of the chorion closely invest the sides of the egg, as is seen in fig. 2, where the chorion of the right side shows. The two halves of the chorion are readily overlooked, but when stained with methylene blue they show as two elongated, pale, greenish-blue, concave plates which together retain the original shape of the egg. Sometimes they remain united posteriorly. The micropyle may remain attached to one side of the split chorion, or else it may break away from the chorion and adhere only to the vitelline membrane (fig. 3, A).

Unlike the chorion, the vitelline membrane is able to stretch considerably and it thus comes to form the only envelope entirely enclosing the egg in later stages (fig. 2). The egg then projects widely from the chorion, especially at the front end, and at this stage it is firm to touch, though the newly laid egg is soft. According to Blunck (1914, p. 86) the vitelline membrane becomes firm consistency owing to the secretion by the serosa of chitin-like lamellae which form a secondary vitelline membrane. This is the serosal cuticle (Wigglesworth, 1950). The vitelline membrane is entirely smooth except

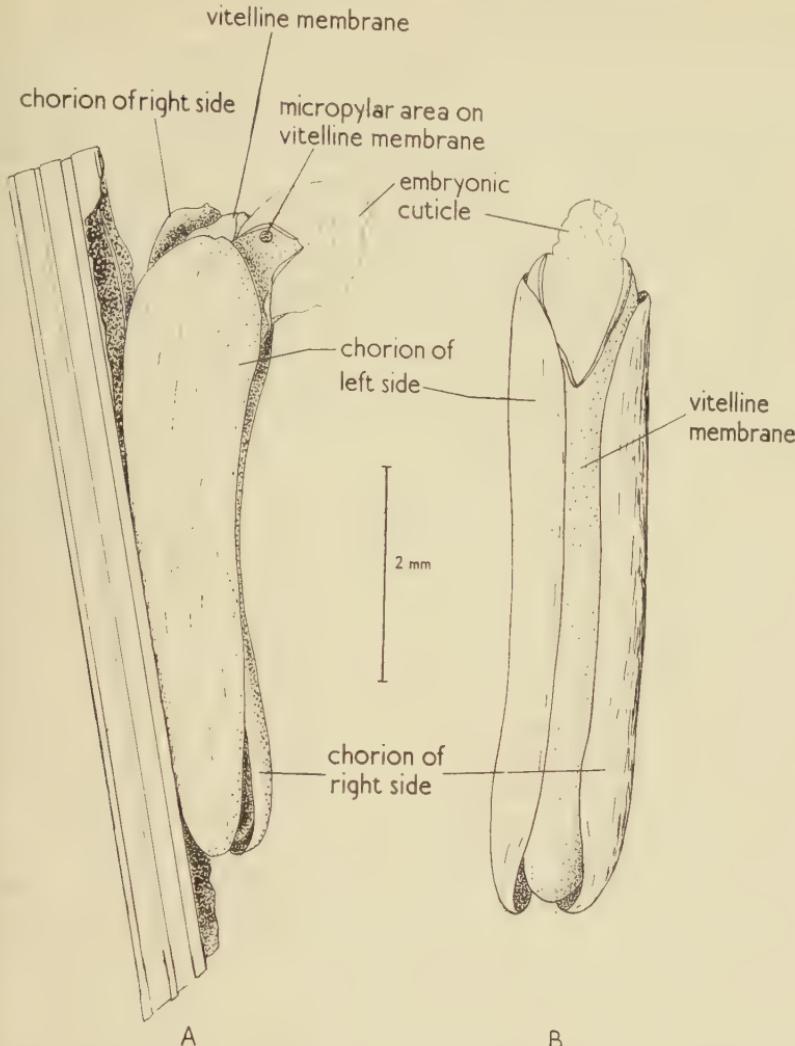


Fig. 3. A, lateral view of hatched egg of *D. marginalis* still attached to leaf of *Carex otrubae*. The egg was lodged between the upper and lower epidermis, which have been torn away. Semi-diagrammatic, as the hatched egg is collapsed and crumpled. B, dorsal view of another hatched egg of *D. marginalis*, showing the chorion split up the dorsal side. Semi-diagrammatic.

The presence of the numerous bacterial cysts in certain areas at the time when the embryo is well formed. The chorion, on the other hand, has a slightly granulated texture owing to a faint embossing of very minute adjacent spots. The chorion stains more deeply than the vitelline membrane with acetocarmine or methylene blue, and sometimes under natural conditions it takes a brown colour from withered leaves surrounding the egg and so appears slightly darker than the rest of the egg. If one removes an egg with an advanced embryo from the cavity in the leaf, the two halves of the chorion are often left behind adhering to the leaf tissue.

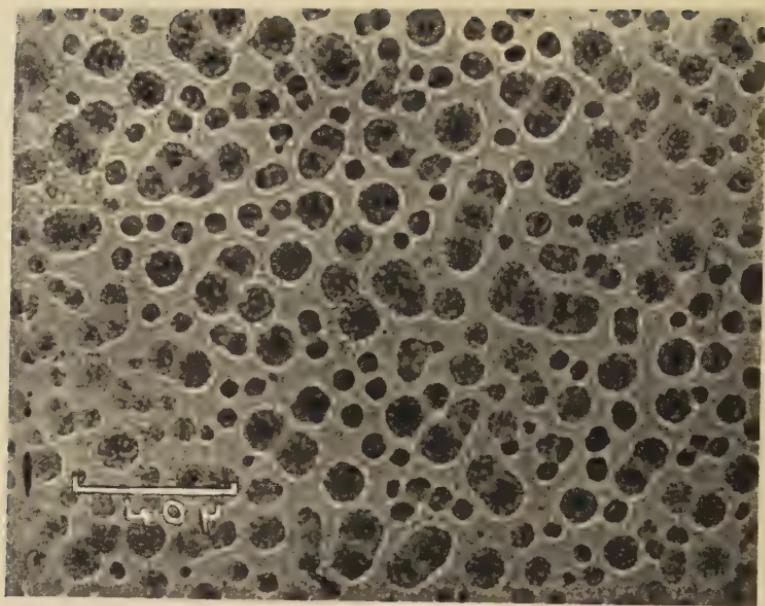
When the larva hatches, it is the vitelline membrane that it ruptures, forming a rent at the anterior pole of the egg, the rent being V-shaped on dorsal surface (figs. 1, A; 3, B). The embryonic cuticle which previously enclosed the embryo, surrounding the antennae, mouth-parts, and legs in individual sheaths, is left partially projecting from the egg as a delicate transparent membrane (fig. 3, A, B). This has already been described (Jackson 1957). The larva then emerges through the widened slit in the epidermis of the leaf left by the beetle in laying. The shell of the egg, distended by larva, contracts after hatching, and the leaf tissue, raised up by the swelling of the egg, collapses, so that hatched eggs are readily overlooked.

It is hoped that the above account, based on the examination of many eggs of *D. marginalis*, will make clear the principal facts regarding the most obvious changes in the envelopes of the egg during development, for so confusion has arisen on this subject in the literature. Wesenberg-Lund (1919) refers to the outer envelope as sticking close to the wall of the egg cavity (*Eiloge*) and tearing as one opens the hole. He looks upon it merely as a lining of the cavity, but remarks that at one end it bears a small knob. I believe this supposed lining to be the chorion and the small knob the micropylar area.

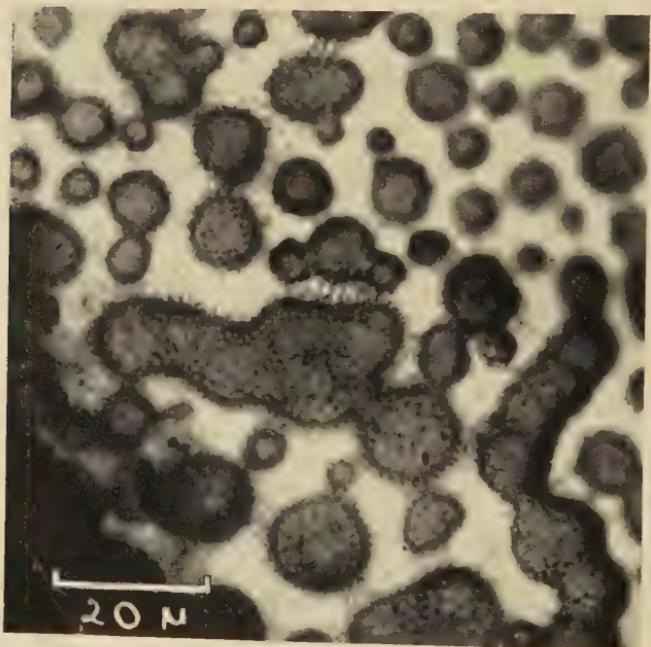
The eggs under observation were laid from the end of March till the end of June in 1957 and 1958. Oviposition in this species appears to be restricted to spring and early summer. The incubation period during May was 177 to 19 days in an unheated north room.

*Location of the bacterial cysts.* The bacterial cysts do not show at all on a newly laid egg or in an egg within 4 days of laying. The exact time of their appearance has not been ascertained, but by the time the embryo is well formed, showing legs and ocelli, they are usually present (fig. 1, B). It is very easy to determine whether they appear on the vitelline membrane before or after the chorion has split. Since the *Dytiscus* egg is so closely enveloped in the plant tissue it will readily be understood that it is by no means easy to ascertain at what moment the chorion splits, for however carefully one removes the epidermis of the leaf to uncover the egg, there is a danger of splitting the chorion in the process. Moreover the bacterial cysts in the early stages are very small and inconspicuous and are not readily seen in a living egg because of the opacity of the embryo, so that it is necessary to remove the egg and to mount the vitelline membrane for microscopical examination. However, in the few young eggs with intact chorion which I have examined I have failed to find the cysts, and I consider that it is most likely that the rod-shaped bacteria gain access to the vitelline membrane after the chorion has begun to crack, and that they then start cyst formation. The bacterial cysts are usually largest and most numerous on the part of the vitelline membrane forming the head of the egg, the area which is exposed by the widening of the initial cut in the epidermis of the leaf and which is left bare when the chorion splits. They also occur all along the dorsum of the embryo and round the posterior pole. They are thus chiefly located on the upper surface of the living egg exposed by the dorsal split in the chorion to the surrounding water and





A



B

FIG. 4  
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osition to receive some light from the splitting of the leaf tissue as the egg ells. They are not present on the ventral surface of the vitelline membrane ich is closely pressed to the leaf tissue. Towards the sides of the eggs, ere the bacterial cysts are few, they are much smaller (3 to 5  $\mu$  in diameter), ile at the anterior pole of the egg they usually attain their largest size (10  $\mu$  diameter), and often occur together in twos or threes or fours (measuring to 36  $\mu$  in length), and they may also form irregular groups (fig. 4, A, B). uring their early period of development on an egg they are small and eley scattered. After the *Dytiscus* larva has hatched, the bacterial cysts rsist on the empty shell of the egg, and indeed appear to increase in size, it is on the hatched eggs that I have found the densest aggregates (figs. 1, 4, A, B). In preparations examined with an oil-immersion lens, Miss Collins s recognized the rod-like bacteria between the cysts.

Often a female *D. marginalis* will drop her eggs in the bottom of the jar stead of inserting them in plant tissue. Such dropped eggs would appear eal to use for the study of the development of the bacterial cysts, but on such gs the slime bacteria never develop. These dropped eggs invariably decay d become mouldy. They are probably infertile, as Joly (1945) suggests.

*Conditions under which cysts of the slime bacteria develop on the egg of *D. marginalis*.* I have found these cysts present on eggs of *D. marginalis* collected om a small pond at Gilston, Largoward, Fife. The eggs had been laid on *Urtica articulatus* L. under water. However, the cysts develop just as readily on gs laid in the laboratory. For my breeding experiments with *Caraphractus* was essential to get the *Dytiscus* females to lay eggs on plants that had not eviously grown in ponds, since plants collected from water might already ntain some stage of the mymarid. I have therefore used plants of *J. articulatus* ted from grassy areas where no water was lying and have grown them indoors of tap water. Moreover I have frequently used the leaves of tall grasses, or of *Carex otrubae* Podh., which had never been in water, and have plunged them in rs of tap-water and introduced to each jar a laying *Dytiscus* female. No soil as placed in the jars. The beetles were fed principally on earthworms. Eggs ere deposited in the leaves under these conditions and usually developed ormally, provided the leaf was not too stiff to allow for the swelling of the gs. The eggs so obtained showed just as abundant colonies of slime bacterial sts as the eggs collected from ponds. It seemed to me probable that the cteria were carried by the beetle herself; so in early December I took a ing female to Miss Collins for investigation, with interesting results.

*Occurrence of bacteria on female *D. marginalis*.* It was found by Miss Collins that slime bacteria are present in enormous numbers on the female ettle. They have been recovered in the rod stage by merely dipping the

FIG. 4 (plate). A, photomicrograph of an area of the vitelline membrane from the hatched g of *D. marginalis* shown in fig. 1, A, with the cysts more highly magnified.  
B, photomicrograph of an area of the vitelline membrane of a hatched egg of *D. marginalis* showing some bacterial cysts in large groups. Preparation stained in aceto-carmine and mounted euparal.

posterior extremity of the living beetle in sterile water. Moreover, by trilling the apex of the abdomen of the female over an agar plate Miss Coll obtained within 24 h swarms of the rod-shaped vegetative cells characteristic of slime bacteria. She obtained the highest numbers of slime bacteria from brushings of the posterior dorsal extremity of the beetle's abdomen in sterile tap-water and the subsequent plating of these on an agar plate. The posterior segments of the beetle are normally covered by the elytra and by dense setae, but the beetle protruded these segments on being touched. If rod stages of the bacteria were recovered from the head and prothorax, which are smooth apart from punctures, and devoid of bristles.

*Effect of parasitism by Caraphractus cinctus on the bacteria.* When I first observed these organisms on healthy eggs of *Dytiscus*, I was puzzled not to have noticed them on various *Dytiscus* eggs which I had mounted which were parasitized with *Caraphractus*. From an examination of many parasitized eggs in preservative or mounted, I have now found that the bacteria fail to develop normally on such eggs. A successfully parasitized *Dytiscus* egg may contain from 25 to 50 imagines of *Caraphractus*, and usually the entire contents of the egg are consumed. Moreover, in eggs with many parasites the vitelline membrane swells so that the chorion splits, just as in an egg in which a *Dytiscus* embryo is developing. It would appear that the bacteria begin to develop on these eggs but find conditions unsuitable and perish. I have frequently found the remains of colonies of bacterial cysts on parasitized eggs, but they are brown and abnormal in appearance. Often only a sprinkling of brown dots may show at the anterior pole of the egg, while at other times the cysts are certainly present, but each cyst is bordered with a brown ring and has not the translucent, greenish appearance of healthy colonies. Only in one parasitized egg I have examined did the organisms appear more nearly normal and the egg contained only 5 parasites of *Caraphractus*; so perhaps the conditions were more suitable to the development of the bacteria.

#### *DYTISCUS SEMISULCATUS* MÜLL

I collected a single female of this species at Tents Muir in Fife on October 1958. It was placed with *Juncus articulatus* and began laying from the end of October till February 1959. The eggs were inserted in the sheath in the basal portion of the *Juncus* leaf and were orientated in the same way as those of *D. marginalis*, with the dorsal surface upwards and the anterior end under the slit made by the ovipositor. It was surprising to find that, contrary to what occurs in *D. marginalis*, the chorion remained in close contact with the vitelline membrane throughout development. In some eggs small irregular longitudinal cracks were observed in the chorion, in others even these did not show and only rarely in a hatched egg did a piece of chorion become partially detached. Hatching occurs by the larva forcing an opening in the anterior end of the egg, through the combined vitelline membrane and chorion. The eggs just before hatching measures from 7.8 to 8.6 mm in length. After hatching the shell contracts by more than 1 mm in length. The larva about an hour

at hatching measures 18 mm, excluding the cerci. The embryonic cuticle gains within the egg but a small portion of it may project from the hole. The incubation period of eggs laid in late October was about 3 weeks.

*Bacterial cysts.* Many eggs laid by this female during the winter have been examined before and after hatching, but only on some eggs were bacterial cysts found and usually these were sparingly present and widely scattered. In no case did they form the even and regular covering as on the egg of *D. marginalis*. Since the vitelline membrane in *D. semisulcatus* is so closely covered by the transparent chorion, the location of the cysts could only be determined by peeling off a strip of the chorion, when the cysts were found on the vitelline membrane below, just as in *D. marginalis*. The few cysts found showed a more definite wall than did the cysts on the egg of *D. marginalis*, but Miss Lins considers that they belong to the same group of bacteria.

### DISCUSSION

It has been shown that in the egg of *D. marginalis*, in which the chorion splits widely long before the larva hatches, the vitelline membrane is closely covered in certain areas with cysts of a slime bacterium. In the egg of *D. semisulcatus*, on the contrary, the chorion closely surrounds the vitelline membrane throughout development, showing usually only a few small cracks; and bacterial cysts, when they are present, are usually few and widely scattered. From this it seems probable that the vegetative stage of the bacterium gains access to the vitelline membrane as the chorion cracks. It is known that the female of *D. marginalis* carries these bacteria in great numbers at the apex of the abdomen and it seems most likely that they are transferred during laying in the gelatinous cement with which the female fastens the egg into the plant tissue. As the chorion splits the bacteria will make their way on to the vitelline membrane and there start cyst formation. This association between slime bacteria and the vitelline membrane is strikingly developed in the egg of *D. marginalis*, probably because the vitelline membrane in this species is so much exposed.

It is too early to make any suggestions regarding the biological significance of this association between *Dytiscus* eggs and slime bacteria. It may or may not be a symbiotic association. In the egg of *D. semisulcatus* the bacterial cysts may not always be present, and when they occur they are usually few and widely scattered; yet the eggs develop normally. The egg of this species is protected throughout development by the chorion, while in the egg of *D. marginalis* much of the vitelline membrane becomes exposed as the chorion splits. According to Bergey and others (1948) the slime bacteria often appear to live in close association with various true bacteria and are probably parasitic on them. There is a possibility that the presence of the slime bacteria on the exposed areas of the vitelline membrane may afford protection against harmful bacteria. It is at least certain that their presence is in no way injurious to the developing embryo.

The fact that the bacterial cysts do not congregate on the chorion, the outer envelope of the egg, even in *D. semisulcatus* where the chorion remains in position, is evidence that the bacteria do not merely use the egg as a convenient surface to squat upon, but that their stance upon the vitelline membrane provides the conditions necessary to them for survival and cyst formation. In this position they will be in close contact, through the vitelline membrane, with the fluid surrounding the developing embryo, and they may benefit from the gases diffusing from the water into the egg during the respiration of the growing embryo. In the egg of *D. marginalis* the cysts occur principally on the vitelline membrane of the dorsal surface of the egg and especially at the anterior pole, and these are the parts of the membrane that are exposed by the splitting of the chorion and which may be better supplied with oxygen than the membrane of the under-surface of the egg, buried in the plant tissue. When the embryo is killed by parasitism of *Caraphractus*, the conditions for the survival of the bacteria are no longer suitable. After the larva of *D. marginalis* hatches, the cysts are still present on the egg and actually occur in denser colonies. Miss Collins has found the rod cells of a myxobacterium present on an old hatched egg of this species recovered from dead leaves of *Juncus articulatus* collected from a pond in December, when the egg would be at least 6 months old.

There can be no doubt that the female beetle herself transmits these bacteria in the vegetative stage to the eggs; how the next generation acquires them remains an unsolved problem. The female *Dytiscus*, according to my observations on captured specimens, may not begin oviposition until probably a year old, so that during many months she will be swimming about amongst aquatic plants where she will doubtless become infected with the rod-like vegetative cells. Were the bacteria carried by the female internally and introduced into the egg during its development within the ovary, one would expect a uniform and constant distribution on each egg, and this is not the case in the egg of *D. semisulcatus*, and not always so even in the egg of *D. marginalis*. The evidence points to an external infection of the egg from the mother, and the degree of infection will be influenced largely by the extent to which the vitelline membrane becomes exposed by the splitting of the chorion, and hence will be less in the egg of *D. semisulcatus*.

It is clear that the relationship of the slime bacteria to the *Dytiscus* egg raises many problems which remain unsolved. One does not know why the exposed vitelline membrane provides these bacteria with suitable conditions for cyst formation nor what stimulus is required to cause the encysted cells ultimately to germinate. One point that could be readily ascertained by entomologists is whether these cysts are equally common on the eggs of *D. marginalis* from other localities. The eggs I have found bearing them were laid by various females collected in Fife, by one specimen from Surrey, and by two from Westmorland. It would also be interesting to know whether they are present on the eggs of all species of *Dytiscus* and how far their occurrence depends on the degree of the splitting of the chorion, for it would seem

in my observations on the eggs of *D. marginalis* and *D. semisulcatus* that extent of the breakdown of the chorion is a specific character.

The identification of the organisms present on the vitelline membrane of *Dytiscus* egg was primarily due to Dr. J. W. G. Lund of Windermere laboratory, who first suggested that they might be bacterial zoogloea. I am much indebted to him for his diagnosis. I am especially grateful to Miss V. G. Hains of the same laboratory for all the trouble she has taken in determining their identity more closely, and for culturing the vegetative cells obtained from the female beetle. She has supplied me with much information and given me references to the literature, and I greatly appreciate all the help she has so kindly given me. I wish to express my thanks to the specialists on algae who most kindly examined some of the material before its identification as a new bacterium; namely, Dr. H. Blackler of St. Andrews University, Dr. Francis Drouet of New Mexico Highland University, Mr. R. Ross of the British Museum (Natural History), and Professor Gilbert M. Smith of Oxford University.

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# The Ontogeny and Comparative Anatomy of some Protocerebral Sense Organs in Notostracan Phyllopods

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With one plate (fig. 2)

## SUMMARY

1. An investigation of the ontogeny of some of the protocerebral sense organs of *Triops* was carried out.
2. Attention is called to the presence of a frontal proliferation zone of great importance in the growth of the nauplius and compound eyes.
3. With respect to the nauplius eyes it is shown that to the small tripartite nauplius eye present at hatching additions are contributed partly by the dorso-lateral lobes of the brain, partly by the proliferation zone. This complicated mode of derivation of the nauplius eye is reflected in the arrangement of the nerves. The so-called upper lateral nauplius eye nerve in *Triops* is shown to be the remnant of the connexion between the nauplius eye and the ganglion opticum of the compound eye, and the evidence suggests that it is not really to be regarded as an optic nerve of the nauplius eye.
4. It is shown that the proliferation zone is mainly or exclusively responsible for the formation not only of the distal part of the compound eye but also of the ganglion layer of the lamina ganglionaris and of the distal part of the ganglion layer of the medulla.
5. The dorsal paired frontal organs as described by Claus (1873) are identified with a group of sensory cells situated above the lateral nauplius eye in the adults of *Triops*, and the ontogenetical processes involved in their dislocation are traced.
6. The group of cells in the neighbourhood of the ganglion opticum supposed by Wenke (1908) and Hanström (1931) to be identical with the frontal organs found by Claus (1873) probably constitute a neurosecretory organ.
7. It is shown that despite great topographical differences the ontogeny of the protocerebral sense organs of *Artemia* in many respects follows the same pattern as in *Triops*.

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## INTRODUCTION

WHEN Hanström (1931) published the results of his investigations of *Lepidurus apus* a coherent picture was for the first time obtained of the protocerebral sense organs of the Notostraca. Previous papers, especially those of Wenke (1908), Holmgren (1916), and Hanström (1926) had made important contributions, but several controversial or obscure points remained. These were to a large extent elucidated in the paper mentioned above, where especially the diagrammatic figs. 1 and 4 give a clear concept of the views on the matter which have since then prevailed. After 1931 Debaisieux (1944) added important new facts on the eyes of *Triops*, but otherwise little has been written about the notostracan protocerebrum and its sense organs.

A comparison between certain aspects of the conditions found among the Notostraca and those prevailing in other Crustacea, however, raises several points which call for a reconsideration of notostracan sense organs. The following ones merit particular attention:

1. Is the complicated innervation of the nauplius eye of the Notostraca merely a secondary phenomenon, due to the large size of the eyes, or has it any deeper morphological significance?
2. Are there any closer morphological relationships between the compound and the nauplius eye in the Notostraca, as suggested by Holmgren (1916)?
3. What are the interrelationships between the two unpaired and the paired nauplius eyes?
4. What becomes of the minute frontal appendages observed by Claus (1873) and interpreted by him as dorsal (paired) frontal organs? Are they really identical with the group of cells close to the posterior part of the lobus opticus, as suggested by Wenke (1908) and Hanström (1931)? If not, what are their relations to other frontal organs, especially those of the Anostraca, and what is the morphological significance of the group of cells mentioned in the previous sentence?

The bearing of some of these questions upon larger issues of Crustacea morphology and phylogeny will be readily understood. They could be partially answered by means of a further study of adult material, but the most important thing seemed to be an investigation of the ontogeny of the protocerebrum and its sense organs. The only previous paper dealing at some length with these matters is that of Claus (1873), where, however, he confined himself to describing the outwardly visible changes from stage to stage. Nothing is known about the internal morphology of the larva and its differentiation during development.

Dried mud from the bottom of pools on the island of Öland was kindly placed at my disposal by Dr. Paul Ardö, and from this mud larvae of *Triops cancriformis* could easily be reared in the laboratory. The present account starts with the recently hatched larva; for owing to the comparatively small number of eggs present in the sample, unhatched embryos were extremely difficult to find in the bottom deposit.

## THE NAUPLIUS EYES

*Early development of the brain and nauplius eyes.* While a comparatively large number of authors have dealt with the ontogeny of the crustacean compound eye, little is known about the formation of the nauplius eye. In the Phyllopoda and Anostraca it is fairly well developed at the time of hatching. Claus (1891) pointed out that it is certainly of ectodermal origin in *Branchipus*, where the nauplius eye of the young larvae only gradually loses its contact with the ectoderm. More recently Weisz (1947) confirmed the presence of the nauplius eye rudiment in embryos of *Artemia* before hatching, but gave no details concerning its derivation. Unfortunately *Polyphemus* is one of the cladoceran genera where the nauplius eye is missing altogether, and consequently the minutely careful investigation of Kühnemund (1929) gives no help in this respect.

My own observations on *Artemia* start at a stage when the embryo is just leaving the outer cyst. According to Weisz (1947) this takes place 6 to 8 h before hatching. In my own cultures development generally is a good deal quicker, and as a rule only a few hours elapse between excystment and hatching.

Two points are important with regard to the nauplius eye rudiment at the time of excystment. First, although at this stage it is still in contact with the ectoderm and receives cells from it, the ectoderm area from which it is being derived is more or less clearly separated from the roughly horseshoe-shaped and continuous area of ectoderm which surrounds it on the ventral and lateral sides and from which the brain, including the lobi optici, is being formed. Secondly, differentiation seems to have proceeded somewhat farther in the nauplius eye rudiment than in the brain proper. Practically all cells at this stage contain yolk granulae with the single exception of the pigment cells of the nauplius eye. The direct ectodermal derivation of these pigment cells can be proved in the embryo where they are in some cases still continuous with the body-wall. Already at the excystment they are, however, notably large and their cytoplasm is finely granulated.

Thus the nauplius eye rudiment in *Artemia* appears to be topographically separate from that of the brain and to precede it slightly in development.

For reasons explained above I had no access to unhatched embryos of *Triops*, but a few of my earliest stages were preserved within an hour after hatching. In these recently hatched metanauplii of *Triops*, the nauplius eye is anteriorly in contact with the integument but is otherwise surrounded by the undifferentiated cells of the brain rudiment. The sensory cells of the nauplius eye itself, however, are somewhat more advanced at this stage (compare fig. 2, B), and this lead in differentiation is still more in evidence in the next stage (stage 2 in the terminology of Claus (1873), which will be used throughout the present paper). At this stage the nauplius eye of *Triops* is composed of the same three traditional parts as are found in the Anostraca and most other Crustacea, i.e. one ventral and two lateral ones.

The brain itself is in contact with the anterior, lateral, and ventral ectoderm from which cells proliferate inwards. Seen in anterior view this part of the ectoderm is clearly marked off from the surrounding area. In outline it is roughly horseshoe-shaped, with the opening upwards and closed by a narrow bar. The lateral parts are the rudiments of the future optic lobes. As will presently be shown they also make important contributions to the nauplius eye complex. In this paper they will be referred to as the dorso-lateral lobes. In the interior their medial surfaces are at least partly in contact with each other and in some places they appear even to fuse. In stage 2 the surrounding ectoderm is already beginning to form the fold which in the adult covers the compound eyes.

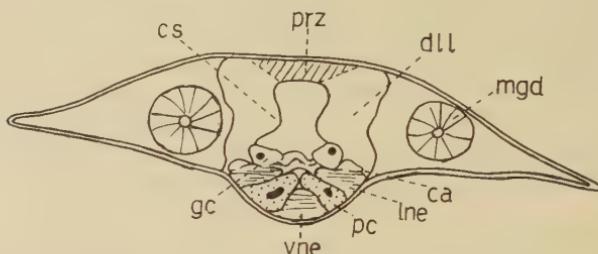


FIG. 1. Semi-diagrammatic sketch of the nauplius eye region of a young larva of *Triops cancriformis* in transverse section, showing establishment of contact area (ca) between dorso-lateral brain lobes and lateral nauplius eyes. The crescent-shaped swellings of the dorso-lateral lobes (cs) are beginning to show. dll, dorso-lateral lobe; gc, giant cell; lne, lateral nauplius eye; mgd, mid-gut diverticulum; pc, pigment cell; prz, proliferation zone; vne, ventral nauplius eye.

During the next few larval stages a series of remarkable transformations takes place in the dorso-lateral lobes of the brain. Already during stage 2 concentration of cells can be seen above the posterior part of the pigment cells of the nauplius eye. They are at least partly derived from the medial side of the proximal part of the dorso-lateral lobes, but to some extent they seem to come from the dorsal part of the protocerebrum proper. They differentiate rapidly and become the medio-dorsal nauplius eye (fig. 2, H, *pne*). Simultaneously in the corner between the base of this medio-dorsal nauplius eye and the dorso-lateral lobes, a couple of very large ganglion cells become distinguishable, the first of the group of giant cells (Holmgren, 1916; Hansström, 1931) located in this area (figs. 1, *gc*; 2, H, *gc*).

Despite the very rapid growth of the hepatic lobes of the mid-gut, which enlarge much more quickly than the brain, the head cavity of the larva becomes more spacious and the different parts of the brain less crowded. The dorso-lateral lobes bend apart and point antero-dorsally and the bridge of cells uniting their dorsal extremities grows in thickness (fig. 1, *prz*). Simultaneously in the antero-medial part of each lobe a crescent-shaped swelling is formed with the convex side inwards (fig. 1, *cs*). It is two or three cells thick, and the cell-

are arranged transversely and are elongated, which is generally a sign of the beginning of fibre formation (Kühnemund, 1929). When the lateral lobes are straightened in the manner mentioned above, the contact between them along the median plane becomes much less intimate. There is a good deal of individual variation, however, and it is doubtful whether the contact is ever completely severed. In one specimen (stage 4) there is only a very narrow connexion between the two otherwise well-separated crescent-shaped swellings; other specimens of similar age the connexion is more intimate. In any case the partial separation is always transitory, for with the growth of the crescent-shaped swellings these bulge more and more and soon re-establish a broad line of contact along the median line.

The ventral end of the antero-medial swelling establishes contact with the dorsal end of the lateral nauplius eye (fig. 1, *ca*), and cells from it begin to range themselves alongside the uppermost sensory cells of the eye and become transformed into sensory cells. This is the beginning of the growth of the lateral nauplius eyes, which gradually transform them into the very large structures typical of the adult (fig. 2, *H, ca*). Some observations indicate that cells from the antero-medial swellings even reach so far in a ventral direction as to coalesce with the ventral nauplius eye, but this I was unable to confirm. Thus the lateral nauplius eyes of the adult are really composite structures, although it is impossible to see any structural delimitation between the different parts, and we find, among adult Notostraca, the unique situation that the upper unpaired and the paired lateral nauplius eyes are to a great extent formed by those parts of the brain which are mainly concerned with the formation of the ganglia of the compound eyes. We shall revert later on to these questions, but before doing so it is necessary to trace somewhat farther the development of the dorso-lateral brain lobes.

*The frontal proliferation zone.* We shall have to consider, at this stage, the changes which have simultaneously taken place in the ectoderm covering the frontal part of the head.

In the recently hatched metanauplius the brain was in the process of sinking into the interior, and the whole of the antero-medial ectoderm of the head was engaged in producing it. Gradually most of this originally well-defined neuro-ectodermal area exhausts its potentialities and both the nauplius eye and most of the brain proper lose their direct connexion with the ectoderm of the body wall. This process, which is in principle a delamination, proceeds gradually from stage 1 to stage 4-5.

There remains, however, a zone of contact and proliferation, which, though greatly reduced in its relative if not its absolute extension, is nevertheless extremely important to the subsequent development. This area covers the bases of the future compound eye of each side (fig. 2, *F, præce*) and forms a comparatively narrow band which unites the anterior parts of two lateral areas (fig. 2, *A, præz*). It thus corresponds to what was previously referred to as the dorsal extremities of the dorso-lateral brain lobes and to the bar connecting them with each other (compare p. 448 and fig. 1). About stage 5-7, however,

the direct contact between this ectodermal area and the dorso-lateral brain lobes has been to a large extent broken over most of the space later occupied by the compound eyes, and the contact between the ectoderm and the brain is mainly confined to a pair of narrow areas on each side of the median line between the compound eyes (fig. 2, A, *prz*).

It should be kept in mind that this frontal ectodermal area constitutes part of the originally much larger one from which the whole brain was derived and that it is the only part of it which permanently retains its contact with the brain. It is, consequently, part of the original neuro-ectoderm, and the only area from which cells from the outside are added to the brain once the earliest stages of larval development have been passed through. It will be referred to as the *frontal proliferation zone*.

It must be understood that the breaking off of the contact between the compound eye part of the proliferation zone and the underlying area that was mentioned above does not take place suddenly. The dorso-lateral lobes of the brain receive cells from these areas at least up to stage 5, and when the anterior medial swellings due to produce the upper parts of the lateral nauplius eyes are first being formed they apparently receive a good deal of material from this area. It is not until the formation of pigment is well under way in the future compound eyes that immigration of cells into the interior of the brain comes to an end in these areas.

FIG. 2 (plate). A, transverse section through left compound eye rudiment of a larva of *Triaenocranum cancriformis*, showing contributions of proliferation zone (*prz*) to distal part of eye (1), lamina ganglionaris (2), and medulla (3). Heidenhain's Azan.

B, medial section through the anterior end of a recently hatched metanauplius of *Triaenocranum cancriformis*, showing one of the pigment cells (*pc*) in direct contact with the integument. The black spots are yolk granules. Note that they are lacking in the pigment cell and most of the surrounding nauplius eye rudiment. Heidenhain's Azan. *br*, brain; *mg*, mid-gut.

C, sagittal section through the head of a half-grown specimen of *T. cancriformis* (length about 8 mm), showing the connexion between the proliferation zone (*prz*) and the compound eye (*ce*) and the lateral (*lne*) and ventral (*vne*) nauplius eyes. *br*, brain. Anterior end towards left. Silver impregnation according to Bodian.

D, *T. cancriformis* of the same size as in C, showing connexion of proliferation zone (*prz*) with lateral (*lne*) and dorso-medial (*pne*) nauplius eyes and with dorsal frontal organ (*dfo*). *br*, brain; *cech*, compound eye chamber. Anterior end towards right. Silver impregnation according to Bodian.

E, transverse section showing topographical relationships between nauplius eye complex (*lne*, *vne*) and dorsal frontal organ (*dfo*) in fully grown specimen of *T. namaquensis*. A pigment cell. Gomori haematoxylin and phloxin.

F, sagittal section through the lateral part of the head of larva of *T. cancriformis* (stage 2), showing splitting of the dorso-lateral lobe in the lobus opticus part (*lo*) and in the later nauplius eye part (*ne*). *ca*, the still rather broad contact area; *prze*, part of proliferation zone forming the compound eye. Heidenhain's Azan.

G, sagittal section of the dorsal frontal organ of a larva of *T. cancriformis*, showing the frontal appendage and sensory cells (*scdfo*). Heidenhain's Azan.

H, horizontal section through the lobus opticus (*lo*) and the lateral (*lne*) and dorso-medial (*pne*) nauplius eyes. The contact area (*ca*) between lobus and nauplius eye is still rather broad. *gc*, giant cell; *pc*, pigment cell. Heidenhain's Azan.

I, oblique horizontal section through the head of a larva of *Artemia salina*, showing the connexion of the proliferation zone (*prz*) of the compound eye (*ce*) with the lamina ganglionaris (*lg*) and medulla (*me*). *mg*, midgut. Heidenhain's Azan.

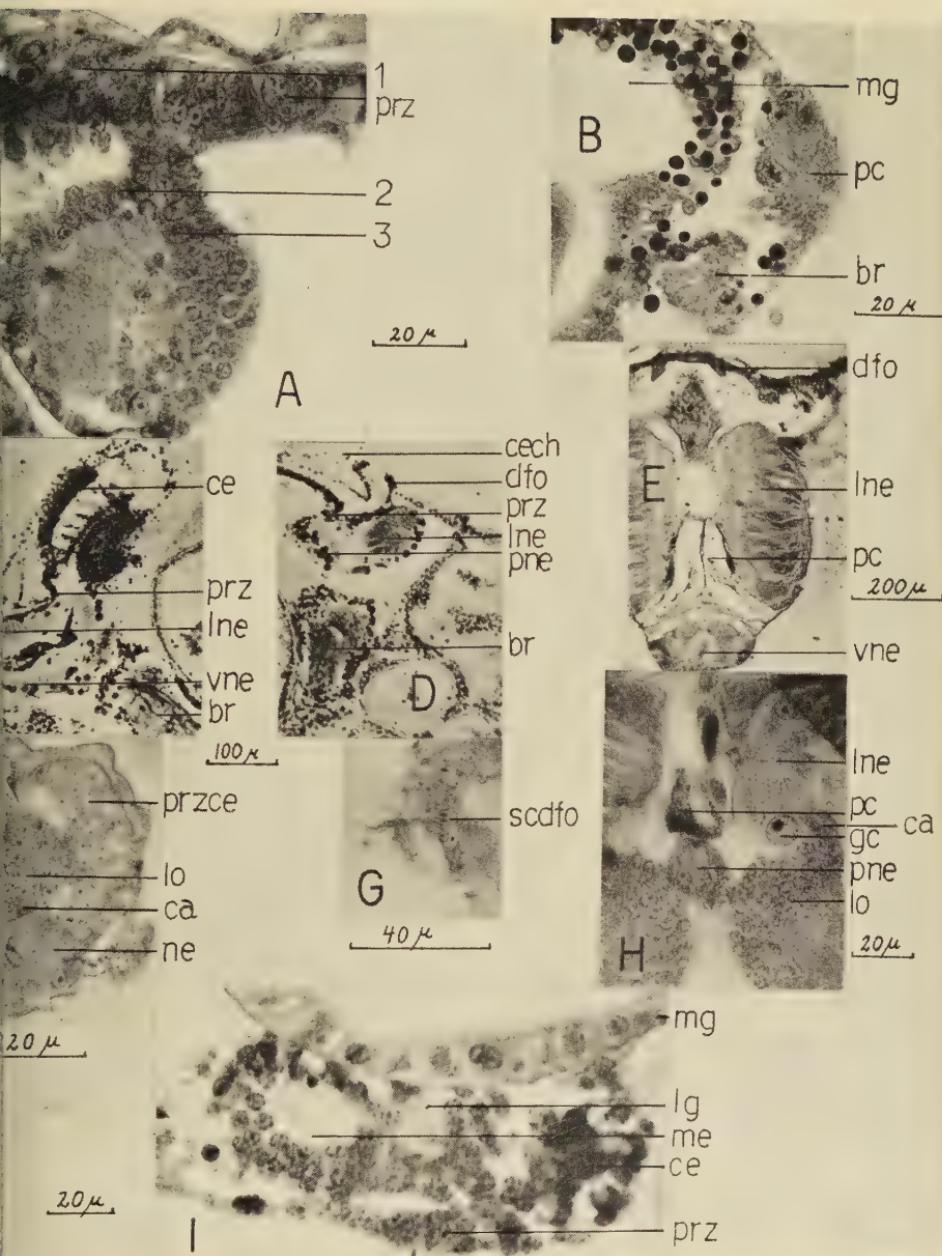
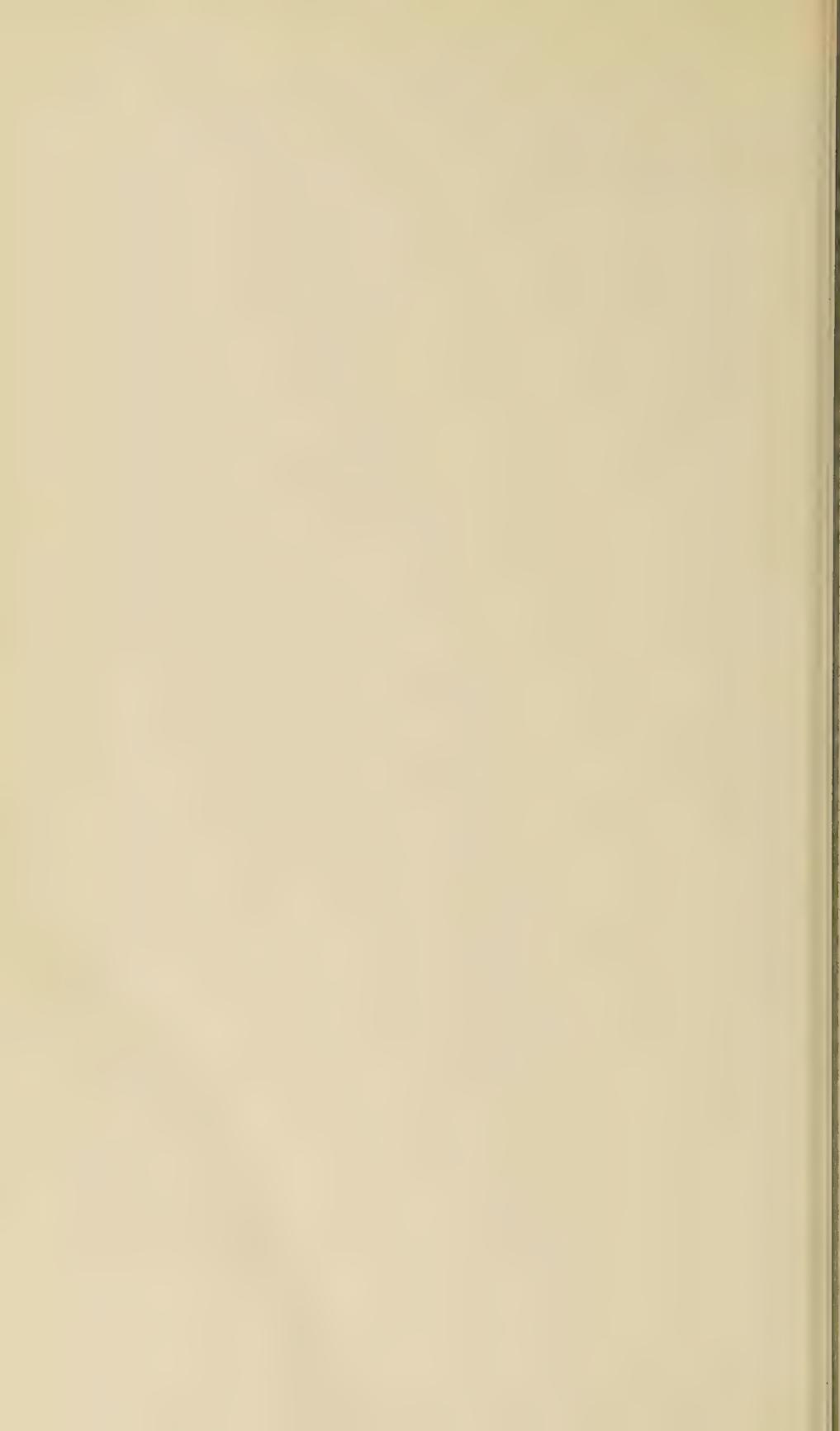


FIG. 2

E. DAHL



Most of the cells of the future proliferation zone apparently originate in the ectoderm in front of the transverse bar (fig. 1, *prz*), mentioned above. There, fairly intense mitotic activity can sometimes be observed, but in the proliferation zone itself there are very few mitoses even during the early part of development and later on there are none. Instead, the proliferation zone is packed with small cells, which later on migrate to different parts of the brain and the sense organs, as will be described in later sections of this paper. The transference of cells from the proliferation zone to their definite sites is a complicated process, the details of which are still to some extent obscure. There appears to be a good deal of active migration on the part of the cells in the proliferation zone, and there seems to be little doubt that the first stages in their movements are active. On the other hand, differential growth also appears to play an important part, for the cells of the organs, of which they are destined to form parts, grow more rapidly than the proliferation zone, and consequently the cells which are on the way to their destination become, comparatively speaking, sucked up and drawn into the various organs, where they themselves start growing.

Gradually, however, the stock of cells contained in the proliferation zone becomes depleted, and in the adult it hardly differs from other parts of the epidermis.

*Longitudinal splitting of the dorso-lateral lobes.* We traced the development of the dorso-lateral lobes up to the time when the antero-medial swellings began to take part in the formation of visual elements of the lateral nauplius eye. Up to this stage the antero-medial swelling is fused to the remainder of the dorso-lateral lobe along the whole of its length, but soon afterwards a longitudinal splitting begins, which, in the end, results in a nearly complete division of the dorso-lateral lobe into the nauplius eye part (which is transformed into sensory cells of the posterior and lateral nauplius eyes) and the optic lobe of the compound eye (fig. 2, F, H). However, there is rarely a complete separation between the lateral nauplius eye and the optic lobe of the compound eye even in the adult. The connexion between the two noted in adults of *Lepidurus* by Zograf (1904), Holmgren (1916), and Hanström (1931) shows a good deal of individual variation, and it can apparently be broken in some specimens (Hanström, 1931), but it is nearly always present. It was interpreted by Holmgren as a nerve coming from the upper half of the lateral nauplius eye. We shall revert to the question of its interpretation in a later section of this paper (p. 454).

The process of separation of the antero-medial nauplius eye part from the postero-lateral optic ganglion part of the dorso-lateral lobe begins approximately simultaneously on the upper and lower end of the lobe. At first the connexion between the two parts is still comparatively broad, but gradually it narrows till only the rather narrow bridge mentioned above remains.

*Contributions of the frontal proliferation zone to the nauplius eyes after the splitting of the dorso-lateral lobe.* It is only owing to the existence of the frontal

proliferation zone that the nauplius-eye complex of the Notostraca attains very large size. As we shall see, the proliferation zone contributes to the growth of all its four main subdivisions, viz. the ventro-medial, the dorso-medial, and the two lateral parts.

As the young *Triops* grows the direction of the length axis of the lateral and ventral nauplius eyes changes: it becomes almost horizontal and parallel to the long axis of the body.

The broadest and most obvious contact is the one between the proliferation zone and the lateral nauplius eyes. Cells from the proliferation zone cross over on a rather broad front to the dorso-lateral margins of the lateral eyes and spread fanwise over the surface, gradually increasing in size both in the ventral and the anterior direction (figs. 2, c, d; 3).

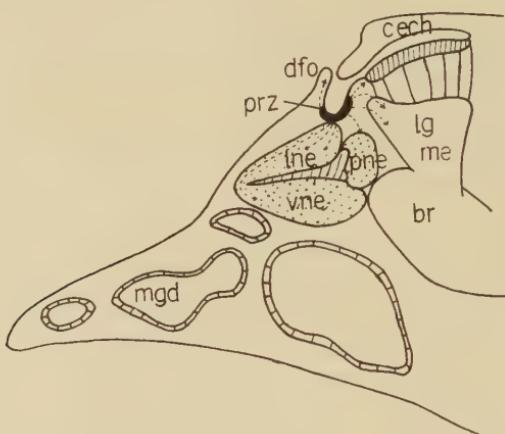


FIG. 3. Diagrammatic sketch of a sagittal section through the head of a half-grown *T. cancriformis*, based upon the sections shown in figs. 2, c and 2, d and adjoining sections. The diagram shows the contribution of the proliferation zone (*prz*) to the growth of various organs as indicated by the arrows. *br*, brain; *cech*, compound eye chamber; *dfo*, dorsal frontal organ; *lg*, lamina ganglionaris; *lne*, lateral nauplius eye; *me*, medulla of compound eye; *mgd*, mid-gut diverticulum; *pne*, dorso-medial (posterior) nauplius eye; *vne*, ventral nauplius eye.

The proliferation zone is connected with the ventral nauplius eye by means of a narrow medial strand of cells which can be traced along the anterior part of the frontal area past the anterior end of the nauplius eye (figs. 2, c; 3). Just there it curves abruptly backwards and joins the anterior end of the ventral eye. Already in the curve the cells begin to increase considerably in size and in the ventral eye itself they grow gradually larger in a posterior direction.

The proliferation zone is connected with the dorsal nauplius eye only by a narrow band of cells which, from the dorsal margin of eye, turns in a ventral direction, while the cells grow gradually larger (fig. 2, d, *pne*). Differentiation of the cells contributed by the proliferation zone increases in a ventral direction.

The connexion between nauplius eyes and proliferation zone is not severed till the specimen is full grown.

*Summary of development of nauplius eyes.* The formation of the nauplius eye complex of *Triops* is a highly complicated process, much more so in fact than is been realized by previous writers. Holmgren (1916) pointed out that the general nauplius eye in *Lepidurus* is to be considered as a structure with a double origin, and Hanström (1931) assumed that the nauplius eye of the early larva of *Lepidurus* is less complicated than that of the adult. Debaisieux (1944) even went one step farther and pointed out that his observations on the growth zones of the nauplius and compound eyes of adult *Triops cancriformis* suggested that a common proliferation zone might provide new material for both types of eyes.

These three writers thus realized the complex nature of the problem and especially Debaisieux predicted fairly accurately in what direction the solution as to be sought. Without access to series of larval material, however, the problem could not be tackled, and as is evident from the descriptions on the previous pages the development is even more complicated than that suggested by Debaisieux.

The nauplius eyes of *Triops* are formed by means of the following three steps:

1. The first formation of the eye from the neuro-ectoderm of the head. The details of this process are unknown since it takes place before hatching and embryological material was not available. At the time of hatching the eye consists of two lateral parts and one ventral part.
2. Growth by means of material derived from the dorso-lateral lobes of the brain. This results in the formation of the dorso-medial (posterior) nauplius eye, the origin of which is paired, and in the very considerable increase in size of the lateral paired nauplius eyes. Possibly the ventro-medial eye is also affected.
3. Completion of the adult nauplius eye complex by means of cells wandering in (or drawn in) from the frontal proliferation zone. This process affects all the four parts and comes to an end only when the specimen is full grown (fig. 3).

In tabular form the origin of the various parts of the nauplius eye complex may be expressed in the following manner:

Part of nauplius eye in adult	Areas contributing to formation of nauplius eye		
	Neuro-ectoderm	Dorso-lateral lobe	Proliferation zone
ventral unpaired	+		
Posterior part		?	+
Anterior part			
lateral paired	+		
Lower (posterior) part		+	+
Upper (anterior) part		+	+
ventral unpaired			+

*Innervation of nauplius eyes.* Hanström (1931) reported the presence in *Lepidurus* of three paired and two unpaired nerves belonging to the nauplius eye complex. The dorsal and ventral medial eyes have one unpaired nerve each, while the lateral eyes have two pairs of nerves which enter the protocerebral nauplius-eye centre from above and, besides, they are connected with the optic ganglia of the compound eyes (compare p. 451). The new facts brought forward here concerning the ontogeny of the nauplius-eye complex call for a few comments on the innervation.

In the adult the nerves of the two unpaired medial nauplius eyes are very large. In the case of the dorsal medial eye we know that it is of a paired origin and to a certain extent this origin is still reflected in the arrangement of the sensory cells, but the nerve shows hardly any trace of it. The ventral medial eye already appears unpaired at the hatching stage, but there is every reason to believe that the first rudiments are paired (compare Hanström, 1926) and in both *Triops* and *Lepidurus* the root of its nerve appears to be paired.

In the case of the lateral nauplius eyes the situation is more complicated. In *Lepidurus* Hanström (1931) found that there are one pair of large, more lateral nerves, and one pair of smaller, more medial nerves, which connect the eyes with the optic centre. A re-examination of these nerves has convinced me that the smaller ones come from the ventral area of each eye, i.e. from the cells roughly corresponding to the part present in the early metanauplius while the larger nerves come from cells which were added later on from the dorso-lateral lobes and the proliferation zone. It is important to note that it receives the centripetal fibres from the whole distal (i.e. dorsal) part of the eyes. There remains the third pair of nerves mentioned by Hanström (1931) which are supposed to connect the lateral nauplius eyes with the optic ganglia of the compound eyes. According to the interpretation of Holmgren (1916) they should pertain to the dorsal part of the nauplius eye, which Holmgren quite correctly as it has turned out, supposed to be a secondary addition. As was already pointed out, this so-called nerve is what remains of the connexion between the upper part of the nauplius eye and the optic ganglion of the compound eye. I do not think, however, that it is really an optic nerve in the strict sense. The connexion seems mostly to consist of connective tissue. There is a great deal of individual variation both in *Lepidurus* and *Triops*. As was pointed out by Hanström, the connexion may not exist at all in exceptional cases. In those specimens where it is to be found it is sometimes a narrow strand of connective tissue including a certain number of nuclei, sometimes a broad zone where the neurilemmas of the nauplius eye and the optic ganglion of the compound eye are in contact and apparently fused with each other. In these cases, however, there is also a direct contact through some opening in the neurilemma. I have, however, been unable to establish one single case where a nerve of the type described by Holmgren really connects the nauplius eye with the optic ganglion. I will not deny that a few nerve-fibres may connect the two parts, but as far as I can see, it is impossible to hold that there is a real optic nerve crossing over from the nauplius eye to the stalk of the compound eye.

In the light of the facts disclosed in the previous sections of this paper there is hardly any reason to expect the existence of such a nerve. The cells from the dorso-lateral lobes of the brain and from the proliferation zone display no outward signs of any differentiation at the time when they become part of the lateral nauplius eyes. It is not until they have reached their definite destination that the last phases of their differentiation begin, including the formation of the centripetal fibres connecting them with the brain. These fibres arrange themselves along the lateral margin of the nauplius eye and merge to form the big lateral nerve mentioned above, which enters the optic centre of the nauplius eye complex. None of them can be seen to break out of this arrangement to cross over to the optic ganglion of the compound eye.

#### ONTOGENY OF THE COMPOUND EYES

Already during the early phases of the longitudinal splitting of the dorso-lateral lobe a certain amount of differentiation is to be seen in the optic ganglion of the compound eye. The ganglion opticum is separated from the external part due to form the crystal bodies and the retinulae (fig. 2, f). The further development of the compound eye in all essentials conforms with the general principles as laid down, e.g. by Bernard (1937), and the final structure of the eye as well as the formation of new ommatidia in the growth zone of the adult were described in considerable detail by Wenke (1908) and Debaisieux (1944). It is hardly necessary, therefore, to enter upon any comprehensive description of the development and structure of the eye. It is sufficient to call attention to a number of new observations made in the course of the present investigation.

The growth of the distal part of the compound eye is accomplished mainly by means of cells from the frontal proliferation zone, which migrate in a lateral direction towards the eyes and in the course of their migration grow and differentiate in the manner described by Debaisieux (1944) for *Artemia* and *Triops*.

Meanwhile the ganglion opticum also continues to grow. Very soon, however, contact is established between the ganglion opticum and the proliferation zone, which apparently contributes a very considerable part of the cellular material included in the ganglion opticum (figs. 2, A; 3). In transverse sections of young specimens about 2 mm in length the relationships are particularly clear. The micrograph (fig. 2, A) illustrates fairly well the lines along which cells from the proliferation zone migrate into the compound eye. These lines are not less than four, two leading into the peripheral part and two into the ganglion opticum. They may be described in the following manner (fig. 2, A):

#### Contributions to the peripheral part of the compound eye

One external sheet of cells spreading laterally immediately below the integument. This sheet apparently produces the corneagenous elements.

Another sheet, immediately below the first one; this produces the retinul and pigment cells.

#### Contributions to the ganglion opticum

To the lamina. One sheet of cells from the lateral part of the proliferation zone grows inwards, at first at right angles to the body-wall but after it has reached the ganglion opticum it is diverted in a lateral direction, producing the layer of ganglion cells covering the distal part of the lamina.

To the medulla. Another sheet of cells similar to the one just described turns inwards from the proliferation zone so close to the other sheet that at first it touches its medial side. On reaching the ganglion opticum, however, it continues to grow straight inwards, producing the ganglion layer covering the distal part of the medulla.

While the important part played by the proliferation zone in the formation of the peripheral part of the compound eye might be expected, its equally important part in the genesis of the ganglion opticum is more surprising. Some of its implications will be considered on pp. 459–60.

#### THE DORSAL (PAIRED) FRONTAL ORGANS AND THE CELL GROUP NEAR THE LOBUS OPTICUS

In his monograph of 1873 Claus described a pair of small frontal appendages in *Triops cancriformis* which he identified as a pair of frontal organs. Some further details were published by Claus in 1886. After that, however, although the frontal appendages were often referred to in papers dealing with Crustacean frontal organs, no new first-hand information was obtained. As the appendages are lacking in adults and also in most of the later juvenile stages it was evident that they are either lost or incorporated in the head.

In the papers of later writers on the brain of the Notostraca a good deal of nomenclatorial confusion prevailed as far as the frontal organs were concerned. Hanström (1931) realized this and introduced logical definitions of the various organs. He believed that the sensory cells of the paired frontal organs found by Claus are identical with a group of cells in the neighbourhood of the lobus opticus of the compound eyes. Because in *Lepidurus* no other organs of similar type were found, this seemed reasonable enough. As will be shown presently my new observations on *Triops* appear nevertheless to call for a reconsideration of these matters.

I found the frontal appendages in some of my slides of larvae of *Triops*, at least up to stage 5 or 6 (fig. 2, G). In other specimens I failed to find them, but as they are very small and exceedingly fragile they are easily lost during handling before embedding, and their absence therefore is doubtless accidental. This was borne out by observations on fresh material.

The sensory cells belonging to each of the appendages number about half a dozen and are situated between the upper (anterior) end of the lateral nauplius eye and the rudiment of the peripheral part of the compound eye. They are typical bipolar cells, and their peripheral fibres are at least 5 times as long

the cell-body and reach to the end of the appendage (fig. 2, G, *scdfo*). The small nerves coming from the groups of sensory cells join the larger (lateral) pair of nerves of the lateral nauplius eye.

Later on the external appendages of the frontal organs are lost. Apparently this reduction is the outcome of a single moult, in the course of which no external appendage is formed in the new integument. The nerve-endings retain their connexion with the body-wall.

At this stage the young specimen gradually loses all its characteristic larval traits, such as the predominance of the antennae over the other limbs, the anterior position of the brain, which becomes entirely encircled by the gut diverticula, and so on. It is thus transformed from a larva into a juvenile specimen, which closely resembles the adult in its structural plan. One of the changes implied and one which is particularly important to the matter in hand is the perfection of the eye-chambers of the compound eyes, by means of a pair of postero-lateral folds which grow in an anterior and medial direction until they cover the compound eyes completely, meet along the median dorsal line of the head, and reach the integument in front of the compound eyes. At the same time the frontal body wall anterior to the compound eyes is folded up into a fairly high and steep fold which is pressed against the anterior wall of the integumental eye-chambers, so that there is only a narrow transverse slit between them (figs. 2, D; 3).

The frontal folding includes the area where the nerve-endings of the frontal organs lie, and so these endings are pushed upwards until they become situated in the dorso-lateral parts of the folds above the lateral nauplius eyes (figs. 2, D; 3). They are present in adult specimens of both species of *Triops* examined by me as a small group of cells immediately below the integument, with nerve-endings in the integument itself (fig. 2, D). From each group a nerve can be traced across the distal end of the lateral nauplius eyes and into the large lateral nerve going along the eye margin towards the brain. Thus the frontal organs are not drawn into the eye chambers of the compound eyes. The large development of the lateral nauplius eye is not compensated by a similar development of the frontal organ and consequently the topographical relationships gradually change. As was pointed out above, the frontal organ of the larva lies between the compound eye and the lateral nauplius eye. With the growth of the latter its relative position changes so that in the adult it lies almost directly above the centre of the lateral nauplius eye and not far from the medial line (fig. 2, E).

The ontogenetical investigation described above leaves no doubt about the entity between the frontal appendages found by Claus and the groups of sensory cells above the nauplius eye in *Triops*. They must be considered to represent the true dorsal paired frontal organs which were previously overlooked in the adult.

The reason why they were not observed previously is quite clear. Both Vanström and Holmgren, who might have been expected to find them as they showed particular interest in this part of the head, worked on *Lepidurus*. In

the slides of adult *Lepidurus* kindly lent to me by Professor Hanström I found no trace of them. Without access to fresh material of *Lepidurus* I cannot say with certainty whether these frontal organs are still present in adults. In any case they are, apparently, not nearly as well developed as in *Triops*.

What becomes, then, of the group of cells near the posterior part of the distal end of the ganglion opticum which Wenke and Hanström suggested might be the dorsal frontal organ? As the true dorsal frontal organ and the group mentioned are both present simultaneously in *Triops*, it cannot be the frontal organ. It seems, in fact, almost certain that it represents a group of neurosecretory cells.

Histologically the cells in the group in the eye-chamber give a very strong impression of being neurosecretory. Their cytoplasm is filled by granules staining red in Azan and dark blue with Gomori haematoxylin. As is well known, such a positive staining with Gomori haematoxylin is not nearly as conclusive a proof of neurosecretory activity in Crustacea as in vertebrates. In *Triops* a very large part of all ganglion cells are Gomori-positive, especially most of the large cells in the ventral chain. Nevertheless, the general appearance of the cells now dealt with, as well as the very numerous Gomori-positive grains in the axons between the cell group and the brain, combine to strengthen the impression that we are faced here by a neurosecretory organ.

#### A COMPARISON WITH *ARTEMIA* AND OTHER ANOSTRACA

A comparison with the genesis of the visual organs in *Artemia* disclosed a considerable degree of similarity.

In larvae of *Artemia* of stage 2 (Heath, 1924) there is still a continuous contact between the neuro-ectoderm and the brain. Later on delamination sets in and a separation of the brain from the integument is brought about. What is interesting from the point of view of the present investigation is that there remains a proliferation zone, or rather several proliferation zones which in some respects resemble that of *Triops*, although they are less uniform in appearance.

In a larva of stage 4 the compound eyes bulge slightly and are provided with pigment. The nauplius eye lies far forward and the ventral side of its ventral component is pressed against the integument, while the anterior parts of the two lateral components touch the frontal integument. There is, of course, no bending upwards of the anterior part of the nervous system as in *Triops*. The proliferation zones (for the ventral nauplius eye divides them into two groups) lie ventrally. At each lateral corner of the ventral nauplius eye lies a group of rounded cells, the task of which is to provide further material for both the medial and the lateral nauplius eye (fig. 4, *przne*). Immediately on the lateral side of this group, yet separated from it by a small notch in the integument, lies the proliferation zone of the compound eye. Its cells are higher and narrower than those of the other group and thus resemble those of the proliferation zone of *Triops*. In a way which appears

be in every respect the same as in *Triops*, it delivers cells for the peripheral part of the eye, as well as ganglion cells for the lamina and the medulla. Observations on early stages indicate that at first these two proliferation zones on each side are connected with each other but that they separate later on. Fig. 4 represents an early stage. In unpublished work on *Artemia* Mr. P. Meurling of the Zoological Institute, Lund, recently demonstrated that there exists a connexion between the optic lobe and the lateral nauplius eye of young larvae, closely resembling that found in *Triops*. Later on it is broken and leaves no traces in the adult. I am indebted to Mr. Meurling for permission to quote this important observation.

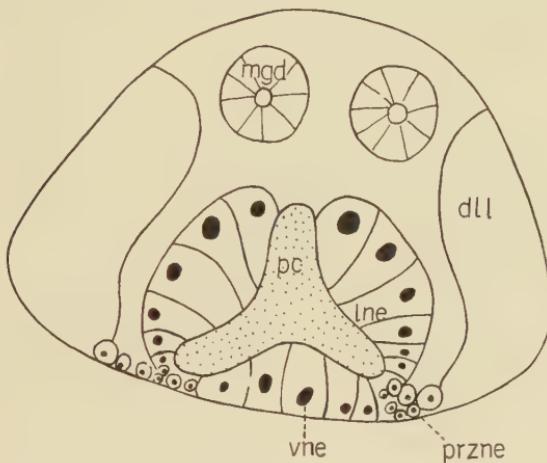


FIG. 4. Diagrammatic sketch of a transverse section through the head of a young larva of *Artemia salina*, showing the proliferation zones of the nauplius eye (przne) in the ventro-lateral contact area between the lateral (lne) and ventral (vne) nauplius eyes. dll, dorso-lateral lobe; mgd, mid-gut diverticulum; pc, pigment cell.

Claus (1886) held the view that ganglion cells for the lobus opticus were derived directly from the ectoderm, not from the brain, though the opposite view has been maintained. Debaisieux (1944, 1952) from studies on adult *Artemia* and *Chirocephalus* arrived at the conclusion that growth of the lobus opticus must take place with the aid of cells from the ectoderm, and the various alternatives suggested by him correspond fairly well with the results of my investigation (fig. 2, I, prz). But the proliferation zone does not provide all the cellular material for the optic lobe. It seems as if at least part of the proximal ganglion cells of the lobe were derived from the brain.

This double origin of the ganglion cells of the lobus opticus gains a good deal in interest when compared with the neurological investigations on *Artemia* carried out by Hanström (1924, 1926). In 1926 Hanström published the semi-diagrammatical sketch of the types of neurones found in the eye-stalk of

*Artemia*, which is here reproduced in fig. 5. He described the neurones in the following manner:

Bei meiner histologischen Untersuchung der Lobus opticus von *Artemia* fand ich . . . dass die kleinen chromatinreichen Ganglienzellen die Verbindung zwischen den beiden Sehmassen vermitteln, indem die distalen (a) [fig. 39 = fig. 5 of the present paper] von der Aussenseite die Lamina durchsetzen um in die zweite Sehmasse, die Medulla, zu enden, während die proximalen kleinen Ganglienzellen (b) die Medulla durchdringen um in die Lamina zu enden. Ausserdem fand ich kleine, chromatinreiche Zellen, deren Verzweigungen nur in der Medulla gelegen waren und demnach als Lokalzellen (c) bezeichnet werden müssen. Die Verbindung mit dem Protocerebrallobus des Gehirns dagegen wird durch die grossen, plasmatischen Zellen vermittelt, welche an der proximalen Seite der Medulla liegen.

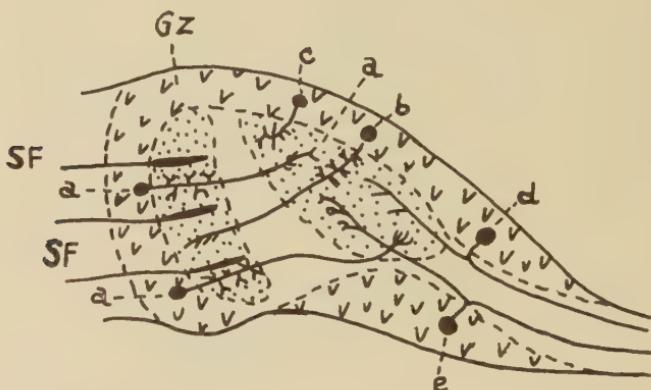


FIG. 5. Arrangement of neurones in the eye-stalk of *Artemia salina* after Hanström (1926, fig. 39, p. 66). SF, fibres from visual cells; GZ, ganglion layer of lobus opticus; a, small neurone connecting the lamina ganglionaris with the medulla; b, a small neurone connecting the medulla with the lamina; c, local neurone of medulla; d and e, large neurones connecting the medulla with the brain.

It is tempting to assume that the small cells in the distal part of the lobus opticus which provide the local connexions within the lobus are those derived from the proliferation zone, while those which establish the connexion with the brain are those from the proximal part of the eye-stalk which were originally derived from the brain rudiment. The histological and ontogenetical facts now available rather support such an assumption, which would bring function and organogenesis into harmony.

A comparison with *Triops* reveals that the proliferation zone of the compound eye has the same function in both cases. Further, the localization of the proliferation zone in relation to the eye is practically the same, although the bending upwards of the anterior part of the whole nervous system in *Triops* brings about a dislocation of the area concerned.

On the other hand, the relationship between the proliferation zone and the nauplius eye seems different in the two genera. In *Artemia*, as was pointed out

ve, the area providing new cells for the nauplius eye lies very close to the proliferation zone just mentioned, but is nevertheless separated from it, and cells are also rather different: they are fairly large and rounded and similar to the neuroblasts of the brain proper. It is also important to note that the addition of new cells to the nauplius eye in *Artemia* takes place at the junction of the ventral and lateral components of the eye, while in *Triops* the growth of ventral and lateral nauplius eye components is effected by addition to the anterior and dorsal areas.

### CONCLUSIONS

In *Triops* as well as in *Artemia* an investigation of the organogenesis of the protocerebral sense organs disclosed much closer connexions between compound and nauplius eyes than were previously known to exist. On the other hand, there is a good deal of evidence favouring the view that the formation of the original tripartite nauplius eye, at the hatching of the metanauplii of both genera, is to some extent independent of the brain and that the closer relations so alluded to above are a secondary phenomenon. At present it is not known whether this comparative independence of the original nauplius eye of the metanauplius has any phylogenetic foundation or whether it is simply an adaptation meeting the need of early function. Until more is known about this, speculation over the matter seems best avoided.

It seems to me a matter of considerable interest that the brain itself through dorso-lateral lobes takes part in the formation of the definitive nauplius eye of *Triops* and *Artemia*. In particular it seems worth noting that neuro-ectodermal cells pass into the brain and from the brain to the lateral and dorsal nauplius eyes and are there transformed into sensory cells. On the other hand, it must also be remembered that the direct connexion between the original nauplius eye and the ectoderm is retained for a long time.

It is also interesting to find that in the Notostraca and Anostraca examined a large part of the cellular material contained in the ganglion opticum of the compound eye is derived not from the brain but directly from the ectoderm of the proliferation zone. To judge by the results obtained by Peabody (1939) on *Idothea* this condition is not probably universal among the Crustacea, but in the case of *Idothea* it is stated (loc. cit., p. 542) that the optic ganglion is formed as a part of the brain. The observations made on the phyllopods also throw an interesting side-light on the much-debated question whether the ganglion opticum acts as an organizer necessary for the formation of the ommatidia (compare Huxley and Wolsky, 1936; Bernard, 1937; Baisieux, 1952). Both in *Triops* and in *Artemia* the distal part of the ganglion opticum is formed mostly if not exclusively by cells directly derived from the proliferation zone, and the cells forming the ommatidia are likewise derived directly from the same zone. But if the generally accepted view that the whole ganglion opticum is formed as part of the brain should be confirmed for, e.g., the Malacostraca, we should be faced by the curious situation that the terminal part of the lobus opticus, supposed to be the active organizer, is formed in

two different ways. In the case of insects there is some experimental evidence against the induction of the ommatidia in the way suggested by Huxley and Wolsky (compare Chevais, 1937), and the new facts brought forward here suggest that the problem should be approached cautiously.

I am convinced that an examination of further Crustacea along the same lines will prove fruitful. Surprisingly little is known about the details of the ontogenetical processes dealt with here, and new facts are needed to provide a more solid foundation for discussions of phylogenetical and comparative anatomical questions, in which the homologies of the protocerebral sense organs necessarily play an important part.

The complex origin of the nauplius eye will have to be considered in connexion with the attempts which are now being made to evaluate its function in various crustacean groups.

I am indebted to Professor Per Brinck for a very large sample of the South African species *Triops namaquensis*, as well as to Professor Bertil Hanström and Professor K. G. Wingstrand for the use of sectioned adult specimens of *Triops* and *Lepidurus*.

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# Some Observations on the Cytology of the Adenohypophysis of the Non-parous Female Rabbit

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With three plates (figs. 3, 4, and 6)

## SUMMARY

Evidence is presented which suggests that in this animal the pars tuberalis may have some function in addition to acting as a bed for the hypophysial portal system, since its cells appear rich in RNA.

The granules of the cells of the pars intermedia are shown to be PAS-positive, but this reaction, unlike that of the basiphil cells of the pars distalis proper and the zona tuberalis, is readily prevented by pretreatment of sections with proteolytic enzymes.

A study of the basiphil cells of the pars distalis proper and of the zona tuberalis, based on cytological characteristics, size, and shape, provides some evidence for the existence of two, and possibly three, kinds of basiphil cell.

The lipid inclusions of the adenohypophysial cells and their relationship to the Golgi elements are described, together with some brief preliminary observations upon the electron microscopic characteristics of acidophil and basiphil cells.

## INTRODUCTION

THE results to be described here form part of an investigation into the cytology of the secretory cycle of the basiphil cells of the adenohypophysis of the rabbit. The cytological criteria of endocrine activity were discussed some years ago by one of the authors (Foster, 1942), and although considerable progress has been made since that time with the advent of new techniques, the assessment of the physiological state of adenohypophysial cells on the basis of their cytological characteristics is still a matter of uncertainty.

The ultimate objective, then, of the present studies was, by using the coital reflex as a stimulus, to try to discover the cytological changes which occur in the gonadotrophic basiphil cells of the female during the successive phases of secretion, discharge, restitution, and storage.

From the outset, certain difficulties were encountered in the initial study of young non-parous oestrous animals to be used as controls for those which was hoped would show post-coital changes. In this work it was decided to use the PAS method for the demonstration of the basiphils since this, having histochemical basis, almost certainly gives more precise information concerning the distribution and granule content of these cells and is, therefore,

preferable to the Azan and Mallory methods used by Wolfe and others (1938), Dawson and Friedgood (1938), and Pearse (1951, 1952b), in their studies of the rabbit hypophysis. Furthermore, it should be pointed out here, that the presence of intracellular granules containing mucoprotein demonstrated by this technique is probably a more reliable means of identification than the use of conventional dyes, since it is open to doubt whether the specific granules of these cells exhibit a true basiphilia at all. In any event the staining mixtures commonly used all consist of acid dyes. The propriety of continuing to apply the classical term 'basophil' (or more usually basophil) to these cells will be briefly discussed later on.

It was found that the fixation of glands by immersion gave unsatisfactory results in that the reaction in many of the basiphils was often very weak and very diffuse so that it appeared probable that the maximum demonstration of the mucoprotein material was not being achieved. This difficulty was to a considerable extent overcome by fixing by perfusion, after a preliminary washing out with 'dextraven' (Allanson, Foster, and Menzies, 1957), this preliminary fixation being followed by immersion of the glands in the same fixing fluid. Subsequently it was found that chilling the fluids to 5° C gave further improvement. It was also noted that fixing by immersion in chilled fixative was a very considerable improvement upon the conventional method of fixing at room temperature.

In the light of what has been said, it seemed desirable to re-investigate certain aspects of the cytology of the non-parous hypophysis, by using the fixation technique outlined above. The following is an account of the results obtained.

#### MATERIALS AND METHODS

Fifty non-parous animals, most of them 5 to 6 months old, were used. The ovaries were always examined in order to assess the degree of sexual maturity.

Perfusion was achieved by cannulation of the left ventricle while the animals were under nembutal anaesthesia. The best results were obtained when the fluids used were chilled to about 5° C and the perfusion carried out at pressures not exceeding 50 mm Hg.

After many preliminary experiments (see Allanson, Foster, and Menzies, 1957), 'dextraven' (5% fructose in dextran, 10% w/v solution) was adjudged the best fluid for the initial washing out of the blood. Although there was some degree of cell-shrinkage in comparison with glands fixed by immersion, this was felt to be more than compensated for by the intensity of the PA reaction obtained in the basiphils of well perfused glands—an intensity rarely obtained when, as in earlier experiments, Ringer's and similar solutions were used.

After the trial of several different fixatives it was found that for general purposes a fluid due to Baker (1944) consisting of a mixture of 10% neutral formalin with calcium and cadmium chlorides (FCC) gave the best results. Glands were, however, also fixed in Helly's fluid, a modified Bouin's fluid.

almi, 1952), Champy's fluid, and Aoyama's fixative. These fixatives, sometimes at 37° C and sometimes at 5° C, were perfused as described above and fixation was completed by immersion at room temperature for about 20 h. In conjunction with the principal staining technique—PAS followed by orange G made up in aqueous phosphotungstic acid, Crossmon's method (1937), Mallory's azocarmine, and Gomori's (1950) paraaldehyde fuchsin (AF) were also used.

In addition, use was made of the following special techniques:

- (a) Aoyama's technique for Golgi bodies followed by toning in gold chloride and treatment with PAS / orange G.
- (b) Baker's (1946) acid haematein test and its appropriate control for the demonstration of phospholipids.
- (c) Sudan black applied to thin frozen sections of material fixed in FCC, for the demonstration of lipochondria.
- (d) The azocarmine method of Dawson and Friedgood (1938) for the demonstration of carminophils.
- (e) Buffered solutions of methylene blue for the assessment of basiphilia in the granules of chromophil cells (Peterson and Weiss, 1955).
- (f) Pyronin and methyl green before and after treatment of sections with solutions of ribonuclease buffered at pH 6.8, for showing basiphilia due to RNA.
- (g) Gram's stain for basiphils (Foster and Wilson, 1952).
- (h) Perfusion with 'dextraven' and 1% osmium tetroxide buffered to pH 7.2 for phase contrast and electron microscopy. Frozen sections of unstained material fixed in FCC were also used for the former purpose.
- (i) The incubation at 37° C of paraffin sections with 0.2% trypsin in tap-water or with 0.2% pepsin in N/100 HCl, to investigate the effect of proteolytic enzymes upon the chromophil granules.

The observations recorded below were made on material fixed by perfusion, unless otherwise stated. For the study of the distribution of the cell types 5  $\mu$  sections were cut in the sagittal or horizontal planes.

## RESULTS

### *General morphology*

The disposition of the various zones of the rabbit's pituitary gland to be referred to in what follows are shown in fig. 1.

The study of the general morphology of the adenohypophysis in material fixed both by immersion and perfusion confirmed the observations of other workers, notably Wolfe and others (1934), Dawson (1937), Green and Harris (1947), and Harris (1947). The most significant property of the pars distalis in this animal is its subdivision into two readily recognizable zones as follows. First, there is the very vascular zona tuberalis, continuous with the pars tuberalis and antero-ventral in position. Histologically this region contains numerous strongly PAS-positive basophil cells, often occurring in groups. Associated with them are apparent chromophobes, both small and large;

acidophil cells are virtually absent except in the neighbourhood of the junction between this region and the pars distalis proper. The second part is the pars distalis proper, morphologically continuous with the pars intermedia where, as pointed out by Dawson (1937), there is an intermingling of cells—pars intermedia cells spreading into the pars distalis and conversely. The pars distalis proper contains abundant acidophil cells which stain readily with

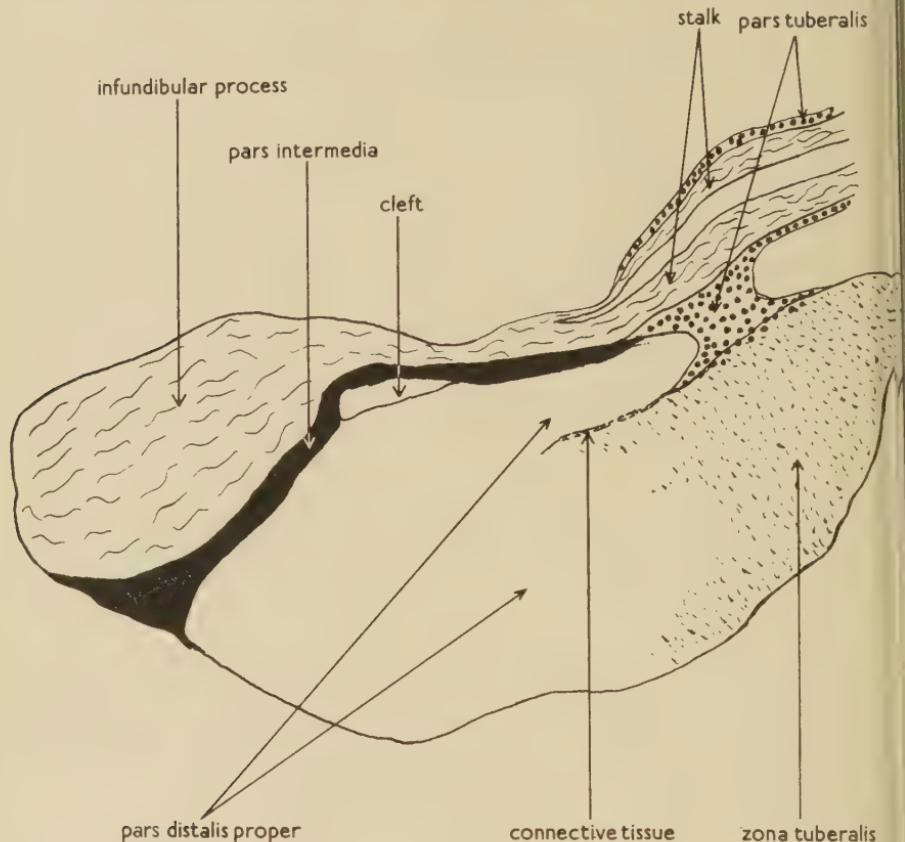
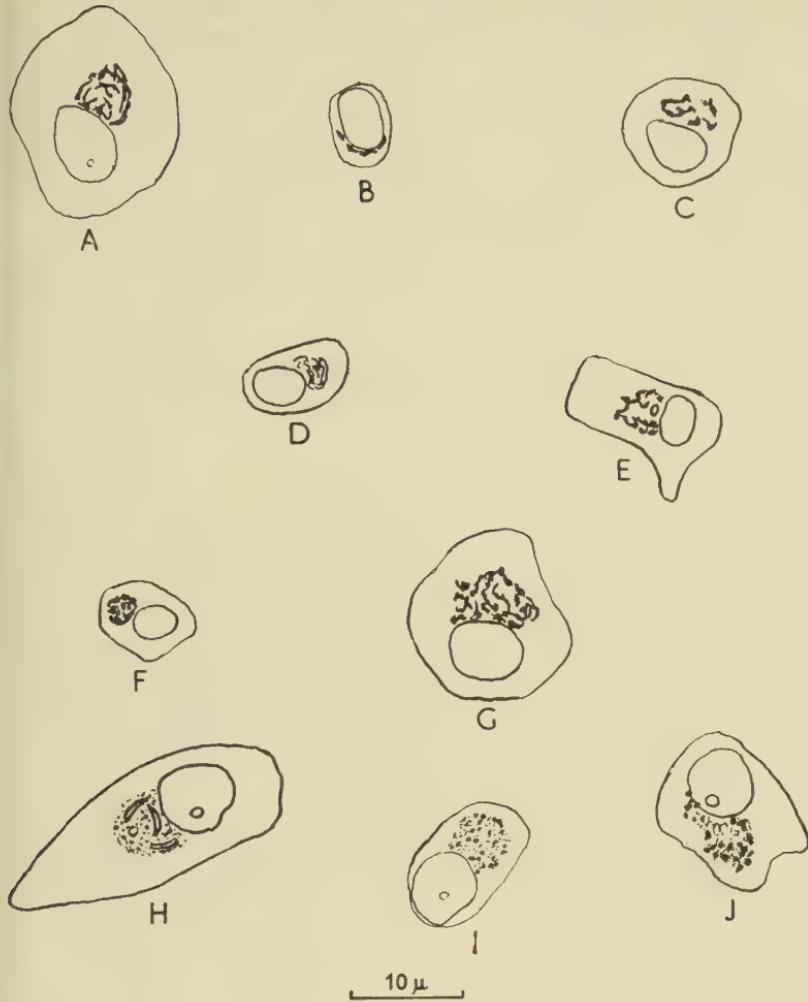


FIG. 1. Diagrammatic representation of a sagittal section through the pituitary gland of a female rabbit.

orange G. The basiphil cells, which give a strong reaction with PAS, are scattered in an apparently random fashion among the acidophil and chromophobe cells. They appear to be less grouped into clusters and, as will be commented upon more fully later, their size range appears to be significantly less than that of their counterparts of the zona tuberalis.

#### *The cytology of the pars tuberalis and the pars intermedia*

As is well known, the pars tuberalis extends from the infundibular stalk to become continuous with the zona tuberalis region of the pars distalis. That part in close association with the infundibular stalk is a very vascular tis-



2. Camera lucida drawings of cells from Aoyama preparations to show Golgi bodies and in black preparations to show lipochondria. A-G, Aoyama; H-J, Sudan black. A, cell from intermedia; B, small chromophobe from pars distalis proper; C, large chromophobe in pars distalis proper; D, basiphil from pars distalis proper; E, basiphil from zona tuberalis; F, acidophil from pars distalis proper; G, large chromophobe from zona tuberalis; H, pars intermedia cell showing fine lipochondria and vacuoles with sudanophil rims; I, chromophil from pars distalis proper showing granular lipochondria and diffuse sudanophilia; J, chromophobe in zona tuberalis showing rather coarse lipochondria, vacuoles, and diffuse sudanophilia.

consists of rather small empty-looking cells arranged in cords and follicle-groups. The examination of sections prepared by the PAS / orange G technique showed a complete absence of basiphil and acidophil cells. Strongly S-positive 'colloid' material was not infrequently seen, however, in the stroma of the follicular groups, although the cells themselves were chromophobe.

In the region distal to the stalk, the pars tuberalis assumes more the

character of the zona tuberalis. The cells are large and basophil cells begin to appear; the cell arrangement, however, is somewhat intermediate in character since follicular groups, sometimes containing 'colloid', are still to be seen. An examination of the Golgi bodies after impregnation by the Aoyama method suggests that this intermediate zone properly belongs to the zona tuberalis since the Golgi material of these cells is much more like that of the zona tuberalis of the pars tuberalis. In the latter, the Golgi bodies appear as rather sparse arrangements of delicate, closely perinuclear strands, in contrast to the large, densely argentophil juxtanuclear nets of the zona tuberalis and the transition region under discussion (fig. 2, E, G).

An unexpected feature of the pars tuberalis was the demonstration of pyronin and methyl green staining of a relative abundance of cytoplasmic RNA. This was generally to be seen as deeply coloured masses in the cytoplasm or as a zone partially investing the nuclear membrane. Control sections incubated in buffer solution alone did not show this ergastoplasmic material (fig. 3, A, B).

The pars intermedia is a very striking region of the rabbit's hypophysis and its cells react very strongly with Schiff's reagent after periodic acid oxidation. Unlike the pars distalis, the reaction is strong even after fixation by immersion. The colour resides in the intracellular granules, which are often numerous and very sharply defined. It was noted, however, that there was some variation among the cells both as to the numbers and intensity of staining of the granules—a fact presumably associated with the as yet unelucidated secretory activity of these cells. Invasive cells from the neighbouring pars distalis were often noticed—basiphilic quite commonly and acidophils less so. It was discovered that the incubation of sections with trypsin or pepsin solutions for quite short periods (about 1 h) virtually abolished the PAS reaction of the intermedia granules, whereas the reactive granules of the pars distalis basiphilic cells were almost completely unaffected (fig. 3, C, D). In this way it was readily possible to locate within the pars intermedia the basiphilic cells of pars distalis origin. It would be possible to use the same technique to discover the exten-

FIG. 3 (plate). A, pars tuberalis. Note dark basophil masses in the tuberalis cells investing the blood vessel in the lower right-hand corner. The infundibular process lies to the left. Fixed in FCC by perfusion and incubated in buffer solution only. Pyronin / methyl green.

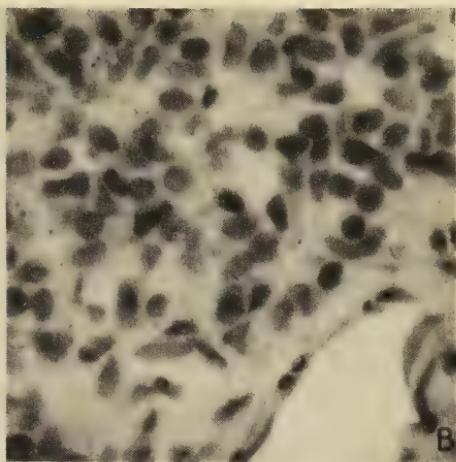
B, pars tuberalis. Neighbouring section to above and stained in the same way after incubation in buffered ribonuclease. Note that the ergastoplasmic material has disappeared.

C, pars intermedia (to the right); the cells react strongly with PAS. To the left a few scattered cells of the pars distalis proper can be seen. Fixed in FCC by perfusion and incubated in tap water at 37° C. PAS / orange G.

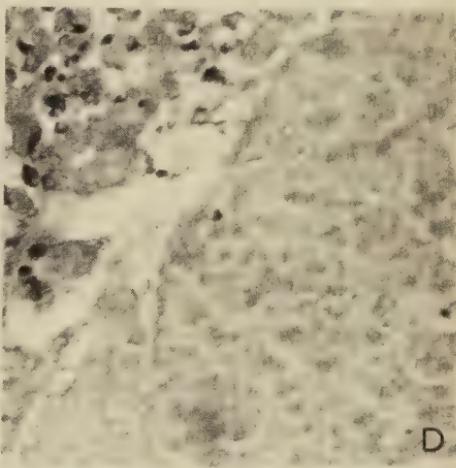
D, pars intermedia. Neighbouring section treated as above after preliminary digestion with trypsin in tap water at 37° C. The PAS reaction of the intermedia cells is destroyed, but that of the basiphilic cells of the pars distalis, some of which can be seen in the top left-hand corner of the photograph.

E, pars distalis proper. The dark cells shown here are acidophils whose granules react positively to Baker's acid haematein test. Frozen section of material fixed in formaldehyde and calcium chloride.

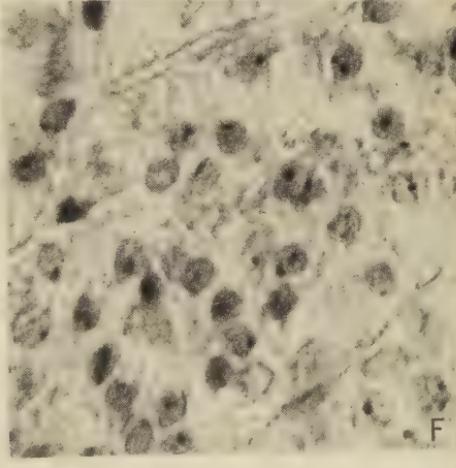
F, section serving as control to above. Extracted with pyridine after fixation of glands in weak Bouin's fluid. Acid haematein.



10  $\mu$



40  $\mu$



10  $\mu$

FIG. 3

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the depth to which intermedia cells invade the pars distalis, as they undoubtedly do. In the present instance, however, this was not attempted. The reaction of the intermedia cell granules to Gomori's paraldehyde-chsin (AF) was somewhat variable, depending very much on its ripeness and the length of staining, but in general the results obtained were similar to those given by PAS.

The Golgi material was demonstrable as very conspicuous spherical or sub-spherical nets, usually touching the nuclear membrane (fig. 2, A). In some cells the argentophil material was more attenuated, assuming the form of a tangle with its base in association with the nuclear membrane. These cells were usually themselves rather elongated in a direction parallel to the long axis of the Golgi body, so that the 'stretching' of the latter might well be of a mechanical rather than a functional nature.

In thin frozen sections of material fixed in FCC and coloured with Sudan black, a well-defined zone of lipochondria could be seen. The individual sudanophil elements were smaller but more numerous than those to be described in the pars distalis. Occasionally the bodies were threadlike or in the form of chains of granules. These lipochondria were embedded, sometimes in association with vacuoles or canals, in a weakly sudanophil background substance (fig. 2, H). This region of the cell corresponded both in position and extent with the Golgi material described above.

After staining with methyl green and pyronin, a considerable number of cells showed cytoplasmic basophilia.

#### *The cytology of the pars distalis*

After the application of the azocarmine technique (as described by Dawson and Friedgood, 1938, and Pearse, 1951, 1952b), to material fixed in formaldehyde / mercuric chloride, two classes of apparent acidophils were sometimes seen—those whose granules coloured predominantly with azocarmine (carminophils) and those staining with orange G. There were, however, cells whose granules were intermediate in colour and, on occasion, groups of presumed basophil cells were observed whose granules had coloured purple instead of blue. Somewhat similar results were obtained in a pituitary taken from an animal 3 h after coitus, where carminophil cells were present in considerable numbers (Friedgood and Dawson, 1938; Pearse, 1951, 1952). Carminophil cells have been described in the pituitary glands of other mammals (Dawson, 1946), and also in the frog (Ortmann, 1956), where it has been demonstrated that they are also PAS-positive and therefore presumably basiphils. In the rabbit, however, this is not so, since the application of the PAS / orange G method after removal of the carmine failed to show a positive reaction, the cells staining with the orange G. In view of the fact that there appears to be an unusually strong subjective element in the azocarmine technique, associated particularly with the preliminary differentiation of the dye with phosphotungstic acid, it was not extensively used, and the PAS method of Pearse (1949) was employed almost exclusively for routine study.

After fixation with FCC by perfusion, a much stronger and more consistent colouring of the basiphil cells with PAS was obtained than after fixation this and other fixatives (Helly, formaldehyde / mercuric chloride) by immersion (compare fig. 4, A, C; B, D). Some shrinkage of the cells did occur, but it was felt that this was less important than an accurate determination of the numbers and position. As was pointed out in a previous note (Allanson, Foster, and Menzies, 1957), there was no evidence to suggest that there was any significant adventitious staining due to a non-specific absorption of dextrans from the fluid used in the preliminary washing out. It is true (Persson, 1952) that when substances like dextraven are injected into the living animal they can be detected in various organs after the lapse of varying intervals of time, but such procedures are hardly comparable with the perfusion of what is virtually a dead animal.

In view of recent investigations, particularly of the rat (Purves and Griebel, 1951 a, b, 1954; Halmi, 1950), which have resulted in the recognition of three classes of basiphil cell, special attention was paid to the position, staining properties, and morphological characteristics of the PAS-positive cells, both within the pars distalis proper and between this and the zona tuberalis. In order to be as objective as possible, camera lucida drawings of 90 basiphil cells chosen at random from the two zones of four different pituitaries were made, along with notes relating to depth of staining, granule size, density, etc. Cells cut through the nuclear region were always chosen for this purpose. In the first place it was clear that there was considerable diversity of shape among the cells, but they could, in general, be divided into two principal classes—those with smooth curved contours and those with straighter angular contours. Intermediate forms were also observed. There was no evidence, however, that cells of a particular shape were restricted to particular areas; all forms were noted both in the pars distalis proper and in the zona tuberalis.

With regard to the size of the basiphil cells, the position was different, since it was evident from casual observation that the zona tuberalis contained cells larger than any to be found in the pars distalis proper (compare figs. 2, D, E; 4, C, D). To investigate this further, camera lucida drawings of nearly 400 cells cut through the nucleus and randomly chosen, were made on squared

FIG. 4 (plate). All fixed in FCC, A and B by immersion; C-F by perfusion after washing through with dextraven.

A, pars distalis proper. The reaction with PAS is so weak and diffuse that definitive basiphil cells cannot be seen. PAS / orange G.

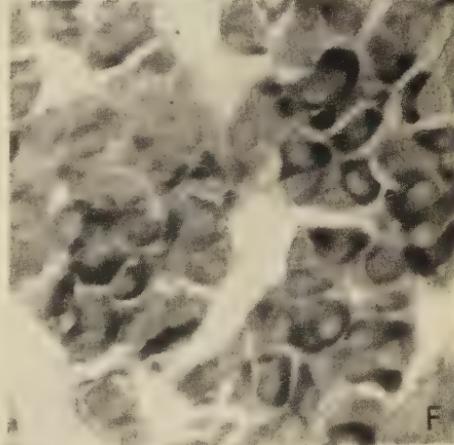
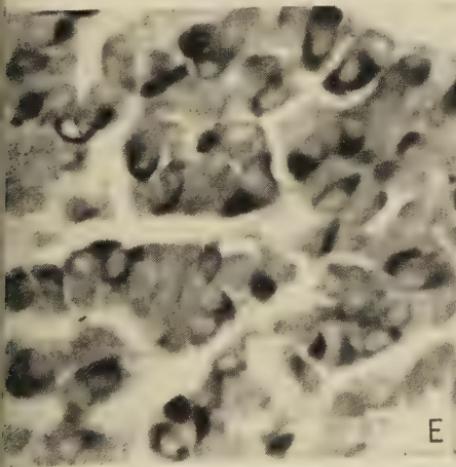
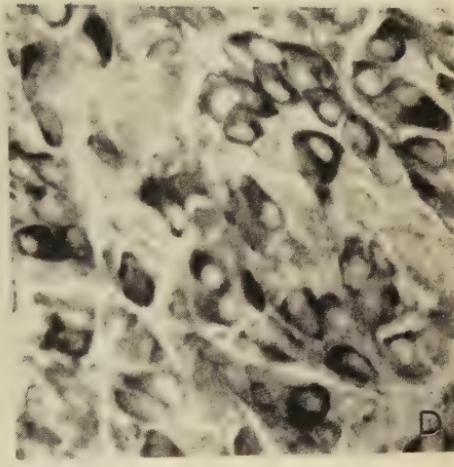
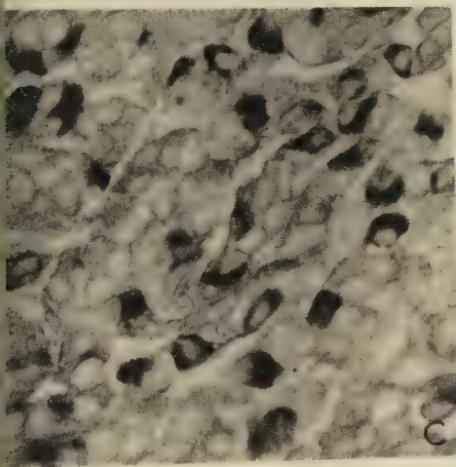
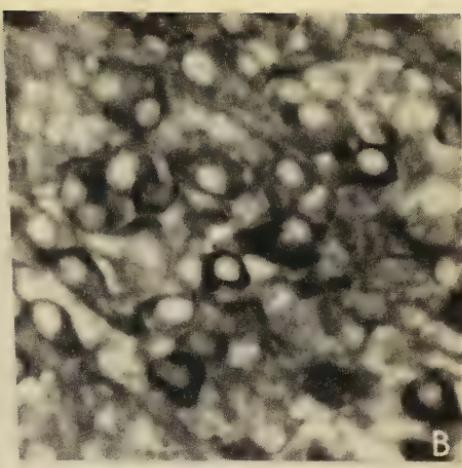
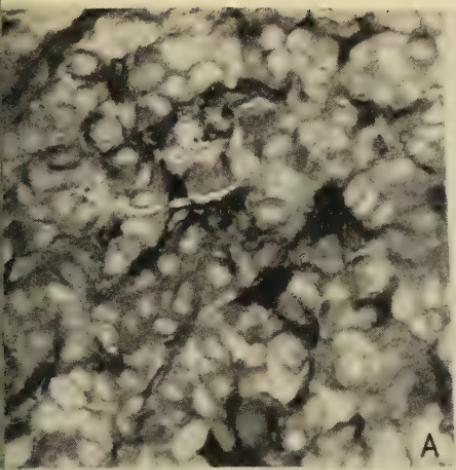
B, zona tuberalis from same gland as above. The reaction with PAS is much stronger, but there are cells giving a diffuse and unsatisfactory reaction. PAS / orange G.

C, pars distalis proper. An intense PAS reaction is given and many basiphil cells can be seen. (Compare A, above.) PAS / orange G.

D, zona tuberalis from same gland as above. An intense PAS reaction is given by the basiphils, which on average appear larger than in C, and are often arranged in groups as in the upper right-hand part of the photograph. PAS / orange G.

E, pars distalis proper. Many basiphil cells stain with aldehyde-fuchsin. Compare with C above.

F, zona tuberalis from same gland as above. Many basiphil cells stain with aldehyde fuchsin. Compare with D.



10  $\mu$

FIG. 4

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per so that their areas could be calculated. For this purpose sagittal sections from a pituitary (PR 48) fixed with FCC by perfusion and from another (PR 49) similarly fixed by immersion were used. The latter gave a PAS reaction sufficiently strong to allow enough basophil cells to be examined and drawn. The number of cells studied from the zona tuberalis and pars distalis

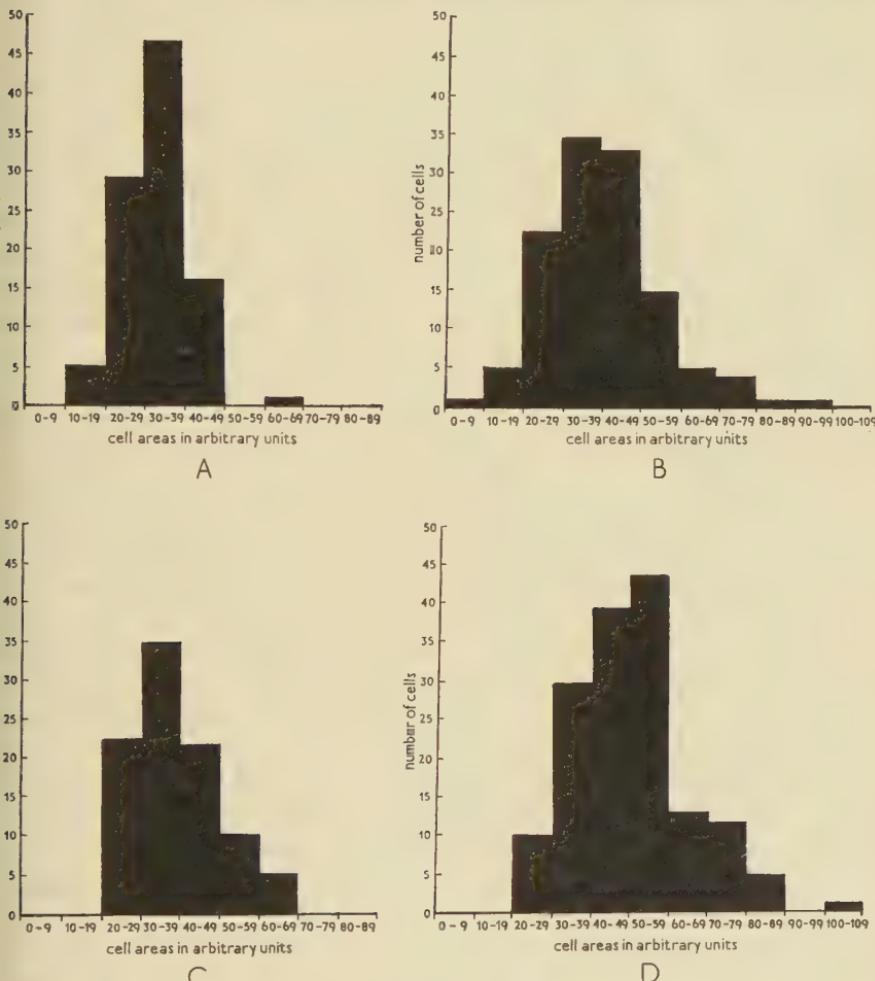


Fig. 5. Histograms showing the size distribution (expressed as cell areas measured in arbitrary units) of the basophil cells of the rabbit's adenohypophysis. A, pars distalis proper; B, zona tuberalis of rabbit PR 48 (preliminary fixation by perfusion); C, pars distalis proper; D, zona tuberalis of rabbit PR 49 (fixed by immersion).

proper of each gland was approximately the same. The results obtained from the examination of these two regions of PR 48 and PR 49 are expressed in the form of histograms in fig. 5.

In both instances, the range of cell size was distinctly greater in the zona tuberalis than in the pars distalis proper, being respectively (in arbitrary

squares) 10-90 and 20-60 for PR 48 and 20-100 and 20-70 for PR 49. When the average cell sizes were calculated for the same two regions they were 36·6 and 32·9 for the former, and 51 and 36·5 for the latter. These results, therefore, limited as they are, support the view that zona tuberalis basiphils tend to be bigger than those of the pars distalis proper. It was further observed that the basiphils of the zona tuberalis commonly occurred in small groups whereas those of the pars distalis proper were more individually scattered among the acidophil and chromophobe cells (fig. 4, c, d).

Analysis of the reaction of the basiphil granules to PAS showed that it was often very intense indeed in many cells after fixation in FCC by perfusion, both in the zona tuberalis and in the pars distalis proper (fig. 4, c, d). The basiphil cells of both zones showed variation in the size and quantity of granules between one cell and another. In the zona tuberalis some cells contained larger granules than any to be observed in the pars distalis proper. On the other hand there were also frequent cells whose granules were so weakly reactive with PAS, or else so scattered, that at low magnification they might be confused with the non-granular chromophobes.

When sections of material fixed either in FCC or formaldehyde / mercuric chloride were treated with Gomori's aldehyde-fuchsin (AF), a positive reaction was observed in many cells, both of the zona tuberalis and the pars distalis proper (fig. 4, e, f). It must be pointed out, however, that several different samples of basic fuchsin were tried before a reliable result was obtained. The distribution of the AF-positive cells closely followed that of the basiphils demonstrated in neighbouring sections with PAS. In order to establish the relationship more precisely, camera lucida drawings were made of small regions containing both AF-positive and AF-negative cells, comprising nearly 400 cells in all. The stain was then removed with weak chlorine water and the sections were treated by the PAS method. It was found that whereas AF-positive cells were invariably PAS-positive (with the exception of one doubtful example), the converse was not true, and this finding applied to both the zona tuberalis and the pars distalis proper. In the 18 fields examined, the proportion of cells both PAS- and AF-positive to those PAS-positive only averaged 61% for the zona tuberalis and 86% for the pars distalis proper. In view of the small number of cells examined, only a general inference can at the moment be made from these figures, to the effect that the basiphil cells which are both PAS- and AF-positive are substantially in excess of the others both in the zona tuberalis and pars distalis proper. It should also be remarked that careful examination has so far failed to reveal any characteristic difference in shape or staining properties between these two apparent classes of cells.

The application of the Gram staining method gave results which were not nearly as consistent as those obtained in the human adenohypophysis after formalin and Helly fixation (Foster and Wilson, 1952), where basiphil but never acidophil cell granules were coloured. In the rabbit, basiphil cells reacted positively only after fixation in Helly's fluid and the results obtained varied greatly as between one specimen and another.

cently (Peterson and Weiss, 1955), evidence has been adduced which tests that the granules of the basiphil cells of 5 different mammalian species (including the rabbit) show a true basiphilia when stained with dilute solutions of ethylene blue at high pH and after fixation in Helly's fluid and further this is distinct from that due to ergastoplasmic RNA. In the present investigation, sections from pituitaries fixed either by immersion in or perfusion by Helly's fluid were stained in the manner described by Peterson and Weiss, and groups of deeply staining cells were drawn with the aid of a camera lucida; the sections were then decolorized in weak acid alcohol and subjected to the PAS / orange G procedure, and the same cells were then re-stained. In only one out of 10 fields examined did the strongly staining cells prove to be basiphils, and even here only a proportion of the total number was demonstrated; in the remaining fields the cells were acidophils, being PAS-positive but positive to orange G. As far as could be made out, the only significant difference in technique between the one described above and that of Peterson and Weiss was in the period of fixation—20 h, as compared with 4 h used by the latter.

As was expected, cytoplasmic basiphilia due to RNA as demonstrated by methyl green and pyronin was found in acidophil, basiphil, and chromophobe cells. Camera lucida drawings were made of nearly 600 cells from sections stained by this method. After removal of the dyes in alcohol, the sections were treated with PAS and orange G to demonstrate the cell types. Analysis of the results obtained showed that of the acidophil cells 31% showed marked cytoplasmic basiphilia, and the corresponding figures for basiphils and chromophobes (large and small) were 64% and 31% respectively. These figures show that a substantial proportion of all these cell classes contain ergastoplasmic material. This is in agreement with observations made upon human material (Foster and Wilson, 1952).

The lipid inclusions of the cells were demonstrated by the use of Sudan black upon thin frozen sections and by Baker's acid haematein test for phospholipids. These methods were applied to both frozen and paraffin sections. Sudan black, after rather prolonged application, revealed numbers of small sudanophil particles or lipochondria in the cells of both the zona tuberalis and the pars distalis proper (fig. 2, I, J). These bodies, like those in the rat, unlike those of man, were restricted to a fairly clearly defined region of cytoplasm, never far away from the nuclear membrane. They appeared to be homogeneous, but on account of their small size any central sudanophobe nucleus would be difficult to resolve. Sometimes the particles were associated with an amorphous sudanophil background material. Small vacuoles (fig. 2, J) were sometimes canal-like structures were noticed in this region, both by direct light and by phase contrast microscopy, but it was not possible to determine whether there was any characteristic relationship between these vacuoles and the lipochondria. Indeed, it was not always easy to be certain whether some of the apparent vacuoles were not really uncoloured secretion granules embedded in the lipochondrial zone. It was noted that this zone

corresponded rather closely in position with the Golgi material as demonstrated by Aoyama's method and thus resembles the condition previously observed in the rat (Foster, 1947).

After the acid haematein test, a large number of cells were found to have reacted positively—their granules having coloured blue-black (fig. 3, E). It was not difficult, after bleaching out the dye with weak chlorine water and then treating the sections with PAS and orange G, to establish that these were acidophil cells. These observations are in agreement with those previously made in the rat (Rennels, 1953) and in man (Foster, 1956).

Aoyama's technique, particularly after initial fixation by perfusion, gave satisfactory impregnation of the Golgi elements throughout the thickness of the specimen, but with some over-impregnation in the extreme peripheral zone. Although the PAS reaction is greatly weakened when used after this method, there is sufficient reaction to enable a considerable proportion of the basophil cells to be identified. The orange G stained the acidophil cells with much difficulty.

With the exception of the small chromophobes, the Golgi elements of the chromophil and large chromophobe cells were almost exclusively in the form of argentophil nets, spherical or subspherical in form and normally in contact to a variable extent with the nuclear membrane. In the pars distalis proper, although unlike the rat and certain other species, there seemed little difference between the form of the Golgi elements as between acidophil and basophil cells, the degree of impregnation was, in general, greater in the former (fig. 2, D, F). The Golgi bodies of the basophil cells of the zona tuberalis were always larger and more heavily argentophil than those of the pars distalis proper. They were in the form of juxtanuclear nets associated with which were rather large argentophil granules (fig. 2, E). Although there is no doubt that the Golgi material is absolutely larger in these basophil cells than in those of the pars distalis, there is some doubt as to whether they are relatively large since the cells themselves are substantially bigger. As was mentioned earlier, acidophil cells are infrequent in this zone, but their Golgi bodies are similar to those in the acidophils of the pars distalis proper.

The small chromophobes of the pars distalis proper contain Golgi elements which are usually in the form of strands and granules closely applied to the nuclear membrane (fig. 2, B); in the large chromophobes the argentophil material is more dispersed and extensive, and resembles more that of the chromophil cells (fig. 2, C). The large chromophobes of the zona tuberalis contain very heavily impregnated Golgi material in the form of large spherical or subspherical aggregates of threads and granules. These appeared to be among the most heavily argentophil objects in the whole gland (fig. 2, G).

Phase contrast examination of thin, unstained, frozen sections of material fixed in FCC and mounted in Farrants's medium showed apparent canalicular systems fairly frequently. These were likewise observed in the form of the so-called 'negative images' of the Golgi element in stained sections—particularly in acidophil cells. In thin sections of material fixed in osmium tetroxide



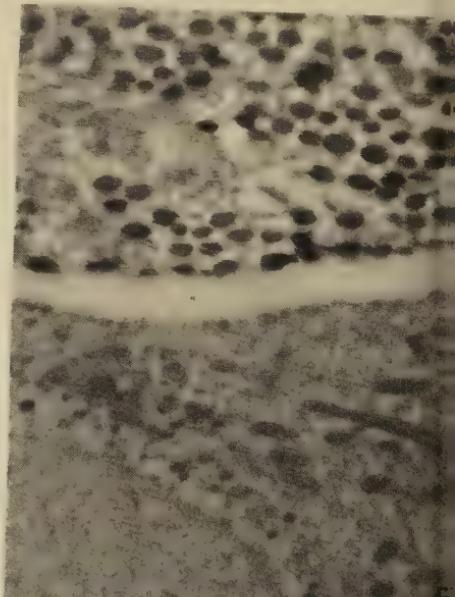
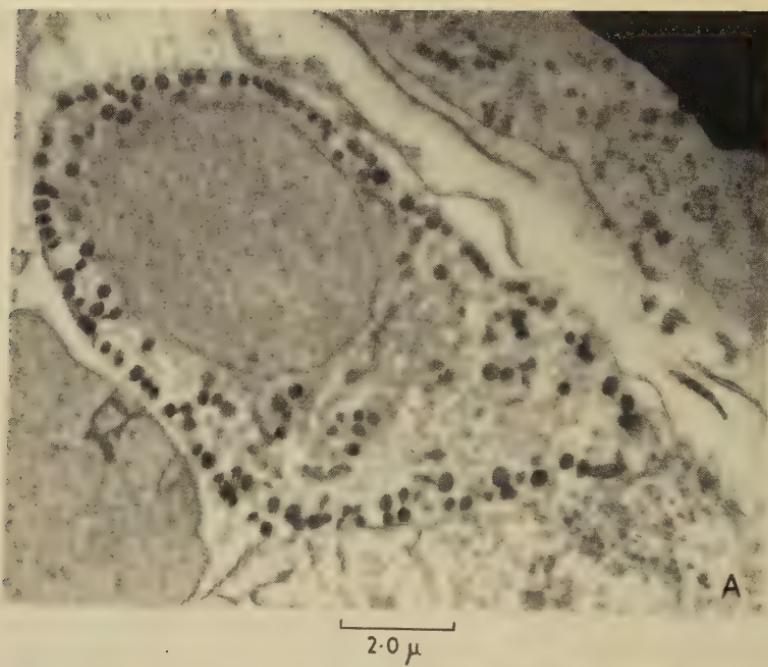


FIG. 6

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buffered to pH 7.2 and similarly examined, canalicular systems were not nearly so evident and, where observed, were much more delicate than after FCC fixation.

A study by phase contrast microscopy of the intracellular granules in sections of material fixed in the manner already mentioned and similarly mounted did not give any very definite information. After fixation in FCC, a large proportion of cells appeared granulated but it was not readily possible to differentiate between acidophils and basiphils. In some sparsely granulated cells it is likely that the objects seen were mitochondria rather than secretion granules. The examination of material fixed with buffered osmium tetroxide showed sharply defined granules of a fairly uniform size in a much smaller proportion of cells, whose arrangement in largish groups suggested that they were acidophils. In view of the fact that such granules have been shown to contain phospholipid, this appears to be a reasonable suggestion. It was not possible to resolve the contents of the remaining cells, although mounting media of different refractive indices were used.

Although phase contrast microscopy and standard staining methods were not successful in demonstrating mitochondria, preliminary study with the electron microscope showed them as fairly dense rod-shaped bodies (fig. 6, a). Evidence of internal cristae was obtained in some instances (fig. 6, c) but these appeared to be somewhat vesiculated, possibly owing to the fact that in order adequately to preserve the glycoprotein of the basophil cells, the material had been perfused with dextraven before perfusion fixation with osmium tetroxide. The granules of the acidophil cells (fig. 6, a, b), like those of the rat (Rinehart and Farquhar, 1953), were very much larger than those of the basiphils (fig. 6, b) and were, perhaps, more opaque; it was not unusual to find a row of the former arranged in orderly array just inside the cell membrane (fig. 6, a), as has also been described in the rat by Green and Van Breemen (1955). Quite often the acidophil granules appeared to consist of a lighter core surrounded by a denser rim (fig. 6, a, b). Since only a limited amount of material has so far been examined, it is not proposed to say anything further at this stage about the results of electron microscopic examination. It is hoped that a fuller report will be published separately later on.

In conclusion, it will be remembered that treatment of sections with proteolytic enzymes for short periods prevented the subsequent reaction of the

FIG. 6 (plate). All electron micrographs of pars distalis tissue, fixed by perfusion with buffered osmium tetroxide solution at 5° C after washing through with chilled dextraven.

A, an acidophil cell occupies the centre of the field. Many of its granules are aligned along the cell membrane, and there is a suggestion of a less dense central area in some of the acidophil granules. Adjacent to the lower edge of the acidophil is part of a chromophobe and, to the right of this, is a small portion of a basophil cell containing small granules.

B, an acidophil cell with large granules occupies the upper part of the photograph. As in A, some of these granules appear to possess a less dense central region. The lower part of the photograph contains a basophil cell in which small granules can be seen, both in the cytoplasm and in association with the cell membrane.

C, a small area of the cytoplasm of the basophil cell shown in B. Mitochondria and possibly part of the Golgi zone (top left) can be seen.

pars intermedia cells to PAS, whereas the basiphil cells of the zona tuberalis and pars distalis proper were apparently unaffected in this respect. More prolonged treatment with pepsin and trypsin did, however, destroy the PAS reaction in many of the basiphil cells of these two regions. At first sight it appeared as if the effect was primarily in the pars distalis proper, but closer inspection showed, first, that some basiphil cells in the peripheral region of the latter zone were in fact relatively unaffected, and that secondly, basiphil cells towards the centre of the zona tuberalis no longer reacted to PAS. It appeared, therefore, that the more superficial cells of both regions were protected from the action of the enzymes, probably owing to the fact that they are the first to be reached by the fixative in which the glands were immersed after their primary fixation by perfusion. Acidophil cells were, judging by their staining capacity, less affected by these enzymes, although pepsin was more destructive than trypsin in this respect.

### DISCUSSION

It is not immediately obvious why there should have been difficulty in demonstrating adequately and consistently the glycoprotein of the basiphil cells of the rabbit pituitary by the PAS method. Since a strong reaction could in fact be obtained in these cells, when successful preliminary fixation by perfusion had been achieved, it seems unlikely that the difficulties encountered were due to a low concentration of these substances in the cells. It would appear rather, that in the rabbit glycoprotein is more labile and is much more readily lost from the cells during the process of fixation by immersion, than in man or the rat, for example. In the former a strong reaction can, in fact, be obtained several hours after death. This view is further supported by the fact that in fixing by perfusion, the nature of the fluids employed in the preliminary washing out was of great importance in determining the end-result. If normal saline or Ringer's solution were used, there was a great loss of reacting material, but this was less evident after isotonic sucrose and less still after dextraven. Furthermore an improvement was observed in fixation by immersion if the fluid used was chilled to about 5° C. In this case, although it is probably true that the fixative enters the tissue more slowly at this lower temperature, it may well be that the postulated diffusion of the PAS-positive material from the cells is correspondingly slowed down to an even greater degree.

In spite of great care taken to ensure, as far as possible, the constancy of the conditions under which perfusion was carried out, variability in the final results was encountered. This was presumably due to factors operating primarily in the pituitary itself, resulting in extreme cases in excessive cell distortion or imperfect perfusion of the gland with a loss of PAS-reactive material in those particular regions. It was apparent, however, that the great improvement over immersion fixation given by this method in successful experiments, compensated for the failures which, in any case, became much less as greater proficiency in the use of the technique was acquired. Baker

1958) has recently expressed the view that in cytology, fixation by perfusion is 'seldom necessary or desirable' because, as he says, in many tissues and organs the amount of fixative held by the vascular system is relatively small and because at the microscopical level a large proportion of the cells will be far removed from capillary vessels. In the pituitary, however, one is dealing with an extremely vascular organ, and this mode of fixation, which is essentially preliminary, since subsequently fixation is continued by immersion, undoubtedly preserves more adequately a cell constituent of the first importance (the glycoprotein), than does immersion alone.

A finding of some interest, yet puzzling, was the discovery in the cells of the pars tuberalis of considerable amounts of basiphil material which could be destroyed by incubation with ribonuclease. This material, resembling in form and distribution the so-called ergastoplasm or chromidial substance, presumably contains substantial amounts of RNA. According to current views this would indicate active protein synthesis within these cells, associated either with growth and multiplication or else with secretory activity. It will be remembered, however, that acidophil cells were absent and that basiphil cells were extremely infrequent and very weakly PAS-positive, so that the cells showing this cytoplasmic basiphilia would be very largely those of the non-granular chromophobe type. This raises the question whether this region of the pituitary may not have some endocrine function. Harris (1955) has suggested that, in the absence of evidence for endocrine function, the role of the pars tuberalis is to act as a bed for the portal vessels. He points out, however, that there is embryological evidence from a few non-mammalian species which would run counter to this view. The absence of visible secretion granules in the pars tuberalis cells does not invalidate the notion of their possessing a secretory function, since, for example, parathyroid cells (with the exception of the oxyphil cells present in some species) are similarly lacking.

The intermediate zone of the adenohypophysis of mammals has been rather neglected from a cytological, and indeed, from a functional point of view. In the rabbit the reaction of the intracellular granules with PAS was intense and appeared to be independent of the kind of fixative used and of the mode of fixation. It is not unlikely that variations in the intensity of the PAS reaction among the cells and differences in the form of the Golgi material may represent different phases of secretory activity. The chemical nature of the PAS-positive material is—as far as the writers are aware—unknown. It is possible that it is a glycoprotein (although the hormone intermedin, which is usually regarded as a specific hormone of this part of the gland, appears not to be (Welsh, 1955)), but in the fixed state it is far less resistant to digestion with trypsin or pepsin than is the glycoprotein of the pars tuberalis and pars distalis proper. Because of this fact, true basiphil cells which have apparently invaded the pars intermedia can readily be detected after such preliminary enzyme digestion. Presumably, therefore, there is some significant chemical difference between the two kinds of PAS-positive substances.

In some respects the observations made upon the pars distalis proper and

the zona tuberalis were disappointing, since the basiphil cells which were particularly studied showed no immediately obvious subdivisions into distinctive cytological categories as appears to be the case in the rat (Purves and Griesbach, 1951a; Purves and Griesbach, 1954; Farquhar and Rinehart, 1954), where three kinds of PAS-positive basiphil cell, producing respectively follicle-stimulating hormone (FSH), luteinizing hormone (LH), and thyrotrophic hormone (TH), have been described. It should be pointed out, however, that the subdivision of the gonadotrophs into those producing FSH and those producing LH has recently been called in question by Barnett, Ladman and others (1956). Although the ultimate test of a cell's function must consist in studying its behaviour in parallel with distinctive functional episodes in the life of the whole organism or during the course of carefully controlled experimental procedures (as has been achieved in the rat (Purves and Griesbach, 1951a; Siperstein and others, 1954)), it had been hoped that some preliminary classification of basiphil cells on morphological, cytochemical, and other grounds would have been possible.

Certain differences did emerge, however, between the basiphil cells of the pars distalis proper and those of the zona tuberalis; the latter tending to be larger in size and frequently aggregated into groups. Also, the impression was obtained that these cells often contained larger and more distinct granules than the basiphils of the pars distalis proper. By contrast, however, there were also cells in the zona tuberalis, which, although apparently chromophophil under the lower powers of the microscope, were seen to contain granules either very scattered and staining normally with PAS, or else fairly numerous but weakly staining with PAS. In the absence of other information, it would appear best, at the moment, to regard these cells as different functional states of the normal zona tuberalis basiphil cell, although their Golgi bodies are, in general, more heavily argentophil than those of any other cells of the adenohypophysis.

The results obtained with Gomori's aldehyde fuchsin provided no evidence for the existence of a specific AF-positive cell with a characteristic shape, as it appears to do in the rat where, according to Purves and Griesbach (1951a, b) and Halmi (1950), it reveals the thyrotrophic basiphil cell. In the rabbit, AF-positive cells of variable shape were found in very substantial numbers both in the pars distalis proper and in the zona tuberalis, and, indeed, many granulated cells of the pars intermedia were reactive to this stain.

The cytological evidence so far obtained would appear to justify the recognition of only two kinds of basiphil cells—those of the pars distalis and of the zona tuberalis, although the weakly PAS-positive, granular cells of the latter zone with their conspicuous Golgi bodies may subsequently prove to be a third. In the pregnant and lactating cat, however, whose adenohypophysis resembles that of the rabbit in possessing an extensive zona tuberalis (Dawson, 1937), Herlant and Racadot (1957) have described three kinds of basiphil cell—all of them PAS-positive, and have respectively ascribed to them luteinizing, thyrotrophic, and follicle-stimulating functions. The first type

of cell ('la cellule rouge brique') is reported as having an additional affinity for orange G and thus masquerades as an acidophil cell in Mallory trichrome preparations. It is further considered by these authors to be the same as the 'carminophil' cell described in the rabbit and cat by Friedgood and Dawson (1938). Such cells were not observed in the rabbit. With regard to the remaining two kinds of basiphils in the cat's adenohypophysis, one—'petites cellules basophiles isolées'—scattered throughout the pars distalis proper, would appear to correspond with the similarly situated and scattered PAS-positive cells of the rabbit. Unfortunately Racadot and Herlant do not specifically state that they observed these cells (which they regard as thyrotrophs) in the non-pregnant animal. The third and last class of cell, considered by these authors to produce follicle-stimulating hormone (FSH), is described as being restricted to the anteromedian part of the gland. These cells seem to correspond in position with the basiphil cells of the zona tuberalis of the rabbit and further resemble them in being the only ones consistently stainable with aniline blue by trichrome staining methods.

With regard to the acidophil cells, as has already been mentioned, some doubt was felt as to whether the carminophils, sometimes demonstrable in non-parous animals after the azocarmine technique, represented a distinct cell category, since the technique was not free from a strong subjective element. These cells (according to Herlant and Racadot (1957) the equivalent of the 'rouge-brique' cells of the cat) have presented difficulties of interpretation for some time, because when Friedgood and Dawson (1938) first described them in rabbit pituitaries, after coitus, and presented evidence suggesting that they were the producers of luteinizing hormone, it was not then known that LH was a glycoprotein, nor were methods for the demonstration of such substances in tissue sections available. As has been pointed out by Pearse (1952), it is extremely improbable on chemical grounds that the carminophil cells produce LH. On the other hand it has, perhaps, been too readily assumed that because the granules of these cells colour preferentially with azocarmine that they must be acidophils. If, however, it is conceded that what have, by general consent, been called basiphil cells are also invariably PAS-positive, then in the frog (Ortmann, 1956), the cat, and a species of bat (Herlant and Racadot, 1957) the carminophil cells should be regarded as a special class of basiphil, since they appear also to be PAS-positive. This point was also investigated in the present work, but all the carminophils examined proved to be PAS-negative yet readily colourable with orange G. It was thus concluded that these cells were acidophils. The fact that these results are in disagreement with those of Herlant and Racadot may indicate a species difference.

The foregoing observations raise the question of the terminology to be applied to what have, by common usage, been called the basiphil cells of the adenohypophysis. It is still by no means agreed that the granules of these cells exhibit, after fixation, a true basiphilia. As was remarked earlier, it was not possible to confirm the observations of Peterson and Weiss (1955), which appeared to show that the basiphil cell granules in the rabbit were truly

basiphil after fixation in Helly's fluid. In view of the differences of opinion which undoubtedly exist (e.g. Moscona and Moscona, 1952), it might well be better to abandon the word basiphil altogether and to substitute 'mucoid' (as has already been suggested by Pearse, 1949) or some similar term indicating to some extent the chemical properties of these cells. The evidence at present available certainly suggests that their glycoprotein content is a much more certain and generally acceptable criterion for distinguishing these granules from others in the adenohypophysis, than is their affinity for basic dyes.

Two kinds of lipid inclusion were demonstrable by Sudan black and Baker's acid haematein. Small granular lipochondria located in the region of the Golgi body were shown by the first method and the granules of the acidophil by the second. These observations are in accord with those previously made on other species (Foster, 1947, 1956; Rennels, 1953; Lacy and Challice, 1957), although Rennels does not state unequivocally whether he believes that the granules he demonstrates are identical with the specific granules of these cells. Recently, Elftman (1958) has questioned the validity of the acid haematein test when applied to cells in which other reducing substances may be present and, on the basis of a process of 'controlled chromation' followed by the application of Sudan black (a method which he believes is more specific for phospholipids), this author describes certain acidophils in the rat 'characterised by the presence of spheroidal granules which differ from ordinary somatotropic granules in staining with Sudan black'. However this may be, there is no doubt that both in man and in the rabbit the granules that are positive to acid haematein are identical with those that colour with acid dyes by ordinary methods. In conclusion, it might tentatively be suggested that the darker peripheral region observed in some acidophil granules by electron microscopy represents the phospholipid component demonstrable by acid haematein in frozen sections, although it must be admitted that conventional microscopy did not suggest a duplex structure for these granules in the latter type of preparation.

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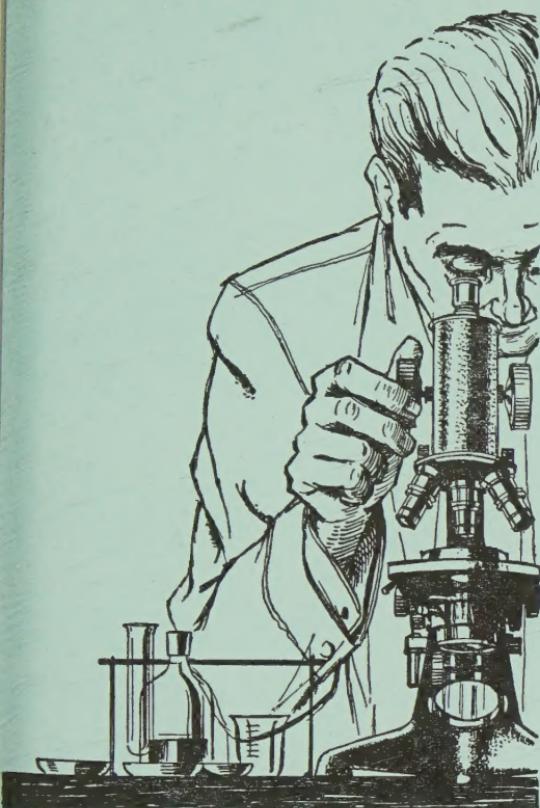
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